CYTOKINE REGULATION OF IMMUNOGLOBULIN ISOTYPE PRODUCTION

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ABSTRACT

Title of Dissertation: Cytokine Regulation of Immunoglobulin Isotype Production

Tina Marie Winderlin McIntyre, Doctor of Philosophy, 1994

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Immunoglobulin (Ig) isotype switching is a process whereby B lymphocytes, initially expressing membrane (m)IgM and/or mIgD, switch, upon activation to the expression of one of 4 IgG subclasses (IgG3, IgG1, IgG2b, IgG2a), IgE, or IgA. The Ig class switch confers a new effector function upon the antibody without changing its antigen specificity thus augmenting the biologic diversity of the humoral immune response.

Previously, cytokines were shown to induce switching to particular Ig classes in activated murine B cells. Thus, IL-4 stimulated IgG1 and IgE switching, IFN-γ selectively induced IgG2a, and TGF-β promoted the switch to IgA. Our studies further elucidated the role of cytokines in Ig class switching by: 1) identifying the

cytokines which functioned as switch factors for IgG3 and IgG2b, 2) establishing the parameters leading to high-rate IgA class switching and, 3) providing additional evidence that the nature of the B cell activator played a determining role in cytokine-directed Ig isotype production.

We determined that IFN-γ induced IgG3 class switching in B cells activated through the mIg, but not LPS, signalling pathway using anti-IgD antibodies conjugated to dextran (αδ-dex) and that TGF-β selectively induced the IgM to IgG2b switch in LPS- but not αδ-dex-activated B cells. This was accomplished by demonstrating: 1) selective stimulation of IgG3 and IgG2b secretion by appropriately activated sort-purified $mIgM^+mIgG3^-$ and $mIgM^+mIgG2b^-$ resting B cells in response to IFN-γ and TGF-β, respectively, 2) cytokine-induced upregulation of the percentage of B cells expressing mIgG3 or mIgG2b, and 3) selective induction of steady-state levels of germline constant heavy $(C_H)\gamma3$ or $C_H\gamma2b$ RNA prior to switch recombination.

Although TGF-β was known to promote IgA switching, investigators were unable to induce more than 1-2% $mIgA^+$ cells *in vitro* using this cytokine. This percentage of $mIgA^+$ cells was far below that found in Peyer's patches (10-15% of all B cells), the major site for IgA switching. We determined a set of parameters (i.e. dual B cell activation, IL-4, IL-5 and TGF-β) which stimulated up to 15-20% $mIgA^+$ cells *in vitro*. These studies thus clarified key issues in cytokine regulation of Ig class switching.

CYTOKINE REGULATION OF IMMUNOGLOBULIN ISOTYPE PRODUCTION

by

TINA MARIE WINDERLIN McINTYRE

Dissertation submitted to the Faculty of the Department of Pathology Graduate Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirement for the degree of Doctor of Philosophy, 1994.

DEDICATION

To my parents,

Joseph and Marcella Winderlin

for all of their love, encouragement, and unfaltering belief in my ability to achieve all of the goals I set for myself, and for instilling in me the value that anything can be achieved through endeavor, perseverance, and hard work.

The greatest achievement of the human spirit is to live up to one's opportunities and make the most of one's resources.

--Vauvenargues

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As so well put by the author, John Ruskin, regarding education:

...It is a painful, continual and difficult work
to be done by kindness, by watching, by warning,
by precept, and by praise, but above all —— by example.

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To improve the golden moment of opportunity,
and catch the good that is within our reach,
is the great art of life.
—Samuel Johnson

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ADCC - antibody-dependent cellular cytotoxicity

αδ-dex - anti-IgD-dextran

AMuLV - Abelson murine leukemia virus

AP - alkaline phosphatase

APC - antigen-presenting cell

BBS - borate buffered saline

BCDF-γ - B cell differentiation factor for IgG(IL-4)

BCG - Bacille Calmette-Guerin

BCGF-I - B cell growth factor (IL-5)

BCGF-II - B cell growth factor (BSF-1, IL-4)

BSAP - B cell lineage-specific activator protein

cAMP - cyclic adenosine monophosphate

CD40L - ligand for CD40 antigen

CDK2 - cyclin-dependent kinase 2

C_H - immunoglobulin constant heavy chain

CMI - cell-mediated immunity

C3b - complement component

DAG - diacylglycerol

DC-PCR - digestion-circularization polymerase chain reaction

DNA - deoxyribonucleic acid

EAE - experimental autoimmune encephalomyelitis

ECF - eosinophil chemotactic factor

EDTA - ethylenediamine triacetate

ELISA - enzyme linked immunosorbent assay

EMSA - electrophoretic mobility shift assay

ES cells - embryonic stem cells

FcεRI - high affinity Fc receptor for IgE

Fcε RII - low affinity Fc receptor for IgE

FcγRII - Fc receptor for IgG, type II

FcγRIII - Fc receptor for IgG, type III

GAF - interferon-gamma activated factor

GAS - interferon-gamma activation site

GαMδ - goat anti-mouse IgD

GM-CSF - granulocyte-macrophage- colony stimulating factor

^{3}H-TdR - tritiated thymidine

HBSS - Hank's balanced salt solution

IFN-γ - interferon gamma

Ig - immunoglobulin

Iγ2b - intronic gamma 2b region

IL - interleukin

IP3 - inositol triphosphate

JAK - Janus kinase

LAP - latency associated peptide

LPS - lipopolysaccharide

MHC - major histocompatibility complex

mIg - membrane immunoglobulin

MOPS - 3-N morpholino propanesulfonic acid

mRNA - messenger ribonucleic acid

MUP - 4-methyl umbelliferyl phosphate

OD - optical density

PCS - protein carrier solution

PFC - plaque-forming cells

PGE_2 - prostaglandin E2

PHA - phytohemagglutinin

PKA - protein kinase A

PKC - protein kinase C

Rb - retinoblastoma protein

rRNA - ribosomoal ribonucleic acid

RT-PCR - reverse transcriptase polymerase chain reaction

S region - switch region

SDS - sodium dodecyl sulfate

sRBC - sheep red blood cell

SSC - sodium saline citrate

SN - supernatant

STAT - signal transducers and activators of transcription

TD - T cell-dependent

TGF-β -transforming growth factor-beta

Th - helper subset of T lymphocytes

TI - T cell-independent

xid - X-linked immunodeficiency

I. INTRODUCTION

PREFACE

Previous studies in the mouse demonstrated that cytokines induced switching to particular Ig classes in activated B cells. Thus both *in vitro* and *in vivo* studies demonstrated that interleukin (IL)-4 co-induced IgG1 and IgE switching (1-16) and interferon (IFN)-γ was a switch factor for IgG2a (17-22). Additionally, transforming growth factor-beta (TGF-β) was revealed as an *in vitro* switch factor for the IgA class although it induced relatively low percentages (1-2%) of membrane (m)IgA$^+$ cells under the conditions tested (23-26). This was far below that observed in Peyer's patches (10-15% of all B cells), the major site of IgA class switching *in vivo* (27,28). In our studies we attempted to answer several key questions regarding cytokine regulation of murine Ig isotype production: (1) Could class switching to IgG3 and IgG2b be positively regulated by cytokines? (2) Were there a set of parameters which could generate high-rate IgA switching *in vitro* at levels which were comparable to that normally observed in Peyer's patches *in vivo*? (3) Did the nature of the B cell activator play a determining role in cytokine-directed Ig isotype production? The following introduction covers three topics related to studies I performed in this thesis: (1) a general overview of Ig class switching including a discussion of the biology of each of the Ig isotypes, (2) a description of the cellular regulation and molecular mechanisms involved in the switching process, and (3) a

discussion of the biology of two cytokines, TGF-β and IFN-γ, which play central roles in the studies described in this thesis.

IMMUNOGLOBULIN CLASS SWITCHING

Immunoglobulin isotypes

Three lines of evidence suggested that B cells producing IgG, IgE, or IgA were derived from cells initially expressing IgM: 1) In 1964 Nossal and collaborators observed that IgM^+ B cells could switch to the expression of another Ig class by demonstrating that upon activation such cells would co-express IgM with at least one additional non-IgM, non-IgD isotype either in the cytoplasm, in culture supernatants as secreted Ig, or on the surface of single cells (29). 2) Administration of anti-μ antibody to chickens and mice early in development prevented the appearance not only of IgM but of all other Ig isotypes as well (30-33). 3) Identical V-region sequences were identified in two monoclonal Ig's, one IgG2 and the other IgM, from a patient harboring multiple myeloma, a malignant plasma cell neoplasia (34).

Thus, an Ig isotype switch occurs when a B cell, initially expressing membrane (m)IgM and/or (m)IgD, switches upon activation to the expression of IgE, IgA, or one of four IgG subclasses. Ig class switching is a highly regulated process such that the induction of particular profiles of Ig isotypes results from (1) the manner in which the particular antigen(s) activates the B cell, (2) the

cytokine-response pattern elicited upon such antigenic challenge, and (3) additional parameters present in the particular microenvironment in which the immune response is occurring. Ig class switching plays a key role in conferring functional diversity to antibodies possessing a particular antigenic specificity during an antigen-driven humoral immune response. Each Ig isotype possesses, through its Fc region, a unique biologic effector function which can include the ability to fix complement, the ability to bind Fc receptors found on a variety of immunologic effector cells, sensitivity to proteolytic digestion, the ability to form aggregates, or the ability to cross mucosal and placental barriers. While the class switch confers a new biologic effector function upon the antibody molecule, it does not change its specificity for antigen.

Although different quantitative and qualitative profiles of Ig isotypes are elicited in response to distinct infectious (antigenic) challenges, the functional roles of particular isotypes in mediating host protection to those infectious agents has been difficult to establish. Although some studies have demonstrated that antibodies of a specific isotype are more efficient at providing protection against certain infectious agents, many studies in various animal models, involving passive transfer of antibodies of different IgG subclasses followed by challenge with infectious agents, have not shown these preferential protective effects (35,36). Studies in humans with specific Ig isotype deficiencies also suggest that no individual isotype is absolutely critical for protection against infection and demonstrates instead the capacity of Ig of a different isotype to

serve a compensatory function (37,38). These studies do not argue against an important role for generation of Ig isotype diversity or the regulation of its induction, but merely emphasize the level of redundancy built into the immune response such that if the expression of *preferential* pathways are diminished or absent, alternative protective mechanisms can serve a compensatory role.

Antibodies of the IgM class characterize the primary immune response that occurs upon initial exposure to an antigen. IgG, IgE, and IgA switching occur later during the primary immune response and account for most of the antibody produced during a memory (secondary) response. Each of the murine Ig isotypes will be discussed below with an emphasis on the structural attributes which endow them with specific effector functions. Such properties make particular Ig isotypes well-suited to protect the host against challenge by certain microbial agents and their products.

IgM Initial antibody responses are primarily of the IgM isotype. The variable regions of IgM antibodies are typically encoded by germline sequences that have not been modified by somatic hypermutation. Somatic hypermutation occurs during a primary immune response, is an active process which leads to nucleotide changes in the V region of a functional Ig gene, and under conditions of antigen selection results in the generation of antibodies with antigen combining sites of higher affinity. This process is termed affinity maturation and often occurs in association with Ig class switching. Thus IgM antibodies elicited during primary

antibody responses tend to bind antigens with low affinity. However, since the IgM molecule exists in pentameric form, and thus has ten antigen binding sites, it can bind with high avidity to multimeric antigens thus compensating for its relatively lower binding affinity (39-42). IgM is highly efficient at fixing complement which allows destruction of microbial cells through opsonization (C3b), complement-mediated lysis (membrane attack complex, C5-9), and the generation of complement components involved in neutrophil chemotaxis (C5a), and induction of inflammatory mediators (C3a and C5a) (43). However, because of its pentameric structure IgM has limited ability to diffuse from sites of local production to distant sites (44). This limitation coupled with its short half-life *in vivo,* compared to IgG, results in IgM antibodies generally being less well-adapted than other Ig isotypes for mounting a protective response to invading pathogens.

IgD Only IgM appears on the surface of immature B cells while both IgM and IgD appear on the surface of mature B lymphocytes. mIgM and mIgD co-expressed by a single B cell have the same amino acid sequence in their variable (V) regions and thus the same specificity for antigen. The selective expression of IgM vs IgD is accomplished by alternative processing of the primary transcript of the rearranged heavy-chain gene (45-48). If cleavage and polyadenylation occurs at the end of the Cμ exon the resulting mRNA encodes IgM, if the site of polyadenylation is further downstream beyond the Cδ exon, conventional splicing will remove all of the intervening mRNA between the V_H-D-J_H exon and the Cδ

exons with the resulting mRNA encoding mIgD. However, upon activation very little IgD is secreted compared to the high levels of secreted IgM often observed. Although the functional role of IgD which is expressed exclusively on the mature B cell, has not been definitively established it has been implicated in playing a special role in antigen-mediated recruitment of B cells and interference with tolerance induction (49-52). Further, mIgD appears to associate with distinct signalling components within the plasma membrane which may mediate the delivery of somewhat different biochemical signals to the B cell than that which occurs upon ligation of mIgM (49,53).

IgE Antibodies of this isotype are uniquely capable of binding to high affinity IgE receptors (FcεRI) on mast cells and basophils. Following the crosslinkage of bound IgE by antigen, these cells are induced to degranulate with the release of inflammatory mediators and produce cytokines. These inflammatory and immune mediators which are released likely play a role in the protective response to helminthic parasites and in the pathogenesis of atopic disease. In particular mast cell-derived mediators may play a role in the expulsion of gut and respiratory tract parasites through stimulation of smooth-muscle contraction and increased vascular permeability(41, 54, 55). IgE-induced mast cell-derived cytokines such as IL-4 and IL-5 (as well as eosinophil chemotactic factor [ECF]) may further magnify the induction of IgE switching and mast cell hyperplasia, and eosinophil recruitment, respectively, after initial induction of such cellular events by T cells (56). Finally, IgE may play a direct role in anti-parasite immunity through binding to

intermediate affinity receptors (FcεRII) on eosinophils and macrophages mediating IgE antibody-dependent cellular cytotoxicity (ADCC) ((57).

IgG subclasses Stimulation by different types of antigen preferentially induces the production of different IgG subclasses: (1) carbohydrate antigens are unique in their induction of IgG3 (58, 59), (2) soluble protein antigens induce predominantly IgG1 (58,60), (3) many different viruses induce primarily IgG2a (61), (4) bacteria often induce IgG2a as well as IgG3 responses (18,62,63), and (5) nematode parasites induce predominantly IgG1 (64). In general, all IgG subclasses have long half-lives, with the exception of IgG3, and are found in high levels in serum. Additionally, all IgG isotypes, unlike IgM, can bind placental Fc receptors which facilitate transport of maternal IgG and afford fetal immunologic protection. While antibodies of the IgG subclasses share many of the same functional properties, specific subclasses perform certain effector functions much more effectively. The distinct functional properties of specific IgG subclasses may enable them to bind particular types of antigen more efficiently or better clear certain types of bacterial, viral, or parasitic infections (41). In a manner much like that of IgE, IgG1 is able to bind mast Fcγ cell receptors and mediate mast cell degranulation thus contributing to their ability to fight nematode parasitic infections (65,66). The ability of IgG1 to be produced in high quantities and its relative inefficiency in fixing complement may make it ideal for neutralizing and clearing bacterial toxins without inflicting complement-mediated tissue damage on the host.

The ability to fix complement via the classical pathway is most effectively accomplished by IgG2a and IgG2b although IgG3 can also activate the complement cascade (67,68). An additional effector function of IgG subclasses involves their targeting of bound antigen via Fcγ receptors for destruction by phagocytosis or ADCC by effector cells. While IgG2a binds FcγRI (on monocytes and macrophages) with high affinity, IgG2b binds, with low affinity, most effectively to FcγRII (on macrophages, monocytes, B lymphocytes, mast cells, and some T lymphocytes) and FcγRIII (on macrophages, neutrophils, mast cells, and NK cells). IgG2a and IgG1 bind both FcγRII and FcγRIII although much less effectively than IgG2b and IgG3 can also bind to FcγRIII (69-72). The ability of IgG2a to effectively fix complement, to bind to the macrophage high affinity receptor for IgG, and to bind Fcγ receptors on NK cells makes this IgG subclass well-suited for conferring protection against viral and gram negative bacterial infections. Although carbohydrate antigens typically induce low affinity antibody responses, IgG3 antibodies, which can readily diffuse into sites of bacterial invasion, are able to self-aggregate after binding the repetitive epitopes present within carbohydrate antigens thus making them highly avid through the process of cooperative binding. Thus IgG3 is well-suited to bind, aggregate, and clear polysaccharide-containing bacteria.

IgA IgA plays an important role in protecting mucosal surfaces from invasion by infectious agents and is the predominant Ig isotype found in external secretions including respiratory, intestinal, and genitourinary tract mucin as well as colostrum, saliva,

and tears. The majority of IgA found in secretions exists in dimeric form associated with secretory component which is a key element enabling transport of IgA across the epithelial lining of exocrine glands into external secretions (73,74). This includes transport of IgA into the gut lumen accomplished by means of an IgA-specific receptor present on the surface of epithelial cells which binds IgA. Together, the receptor-IgA complex is taken into these cells by receptor-mediated endocytosis, transported across the cell, and extruded from the opposite surface into external secretions. Proteolytic cleavage of the receptor as it is extruded from the epithelial cell leaves secretory piece attached to the secreted IgA molecule. IgA has high avidity interactions with viruses and bacteria present in secretions as a result of its dimeric nature, however, this large size does not impede its ability to diffuse through secretions due to the lack of barriers present in this environment. The IgA molecule is also highly resistant to proteolytic digestion which allows this isotype to function in the gastrointestinal tract and other regions where high concentrations of proteases are present. The prevalence of IgA at mucous membranes effects a first line of immunologic defense against invading pathogens and reinforces the physical barrier formed by the mucous layer (78). This occurs, in part, through the binding of oligosaccharide receptors present on the IgA α chain to bacterial fimbriae, as well as specific IgA binding to particular bacterial and viral antigenic components, thus inhibiting bacterial adherence and penetration to mucosal epithelial cell oligosaccharides and other receptor structures (75). IgA activates complement by the alternative, though not the classical, pathway. In

fact, IgA is the most effective isotype at fixing complement by the alternative pathway (76). IgA-mediated complement activation, however, does not appear to play a major role at mucosal surfaces since the level of complement components in secretions is quite low. IgA is also able to bind Fcα receptors present on neutrophils and thus may participate in ADCC (77).

Cellular Regulation

Early studies demonstrated that neonatally thymectomized mice could not respond to the T-dependent (TD) antigen, sheep erythrocytes (79). This supported the hypothesis that the presence of T lymphocytes were essential to promote the differentiation of B lymphocytes into antibody-producing plasma cells following their exposure to antigen. T-dependent antigens are internalized by antigen-presenting cells (APCs) such as macrophages, dendritic cells and B cells, then reexpressed on the cell surface in the context of major histocompatibility antigen (MHC) class II antigens. This leads to cognate interactions between antigen-specific T cells and the antigen-expressing APC. Although soluble protein antigens, could not elicit antibody production in neonatally thymectomized mice such mice responded normally to challenge with purified capsular polysaccharide of Streptococcus pneumoniae type III (80,81). Additionally, antibody responses of congenitally athymic nude mice to pneumococcal polysaccharide were shown to be similar to those of thymus-bearing littermates, whereas, nude mice did not respond to

soluble protein antigens (39-42). Thus, these studies suggested that capsular polysaccharide could elicit antibody production in a T-independent (TI) fashion whereas Ig responses to soluble proteins were strictly dependent upon T cell help (TD). Polysaccharides (TI), unlike soluble (TD) antigens possess highly repetitive chemical structures which allow presentation of numerous identical antigenic determinants to the B cell. This allows polysaccharide antigens to effect multivalent mIg crosslinking, and hence a distinct type of B cell signaling which may account, in part for their relative T cell-independence (83,84). Polysaccharide antigens typically lack intrinsic, non-mIg, B cell mitogenicity and have been defined as TI type II (TI-2) (85) antigens whereas antigens such as bacterial lipopolysacchride (LPS), which are mitogenic for B cells, have been classified as TI type I (TI-1). In any event, the inability of TI antigens to elicit MHC class II-dependent cognate T cell help likely reflects their inability to physically associate with MHC class II molecules. Nevertheless, some form of T cell help has been shown to regulate the antibody response to TI-2 antigens.

Thus, although polysaccharide antigens do not effect cognate T-B interactions, several earlier studies suggested that T cells could enhance IgM to IgG class switching in response to such antigens. Braley-Mullen reported that changing the physical state of the pneumococcal antigen by coupling it to a T-dependent carrier, sheep erythrocytes (SRBC), led to relatively higher levels of production of IgG (86). Vogel and Roberson later showed that mice injected with pneumococcal polysaccharide produced relatively higher levels of

IgG when co-injected with the T cell mitogen phytohemagglutinin (PHA) (87). This enhanced IgG response may have resulted from the PHA-mediated production of T cell-derived cytokines.

These studies set the stage for later independent studies by the laboratories of Severinson and Vitetta which described the ability of certain T cell supernatants, when added to LPS-stimulated B cells *in vitro*, to selectively enhance IgG1 production while inhibiting the synthesis of IgG3 and IgG2b (1-3,88). The activity present in these supernatants was termed B cell differentiation factor for IgG (BCDF-γ). BCDF-γ was later found to be identical to B cell growth factor I (BCGF-I), an activity which enhanced proliferation of anti-Ig-activated B cells, and was named IL-4 upon its cloning (14,15). It was later shown that IL-4 selectively enhanced the synthesis of IgE, as well as IgG1 (13,14,17,303,304).

Further work reinforced the concept that Ig class switching is not a stochastic process but one which can be directed by cytokines. Thus, in addition to the demonstration that IL-4 induces the class switch to IgG1 and IgE, two additional cytokines were shown to selectively induce switching to specific Ig isotypes: interferon-γ (IFN-γ) induced IgM to IgG2a class switching (17-22), whereas transforming growth factor-β (TGF-β) regulated the switch to IgA (23-26). A cytokine which stimulates the production of one or several Ig isotypes also inhibits the production of other Ig isotypes. Thus, IL-4, which induces IgG1 and IgE secretion by LPS-activated cells, inhibits the LPS induction of IgG3 and IgG2b (1,9,13,17,88,89).

IFN-γ which stimulates the synthesis of IgG2a by LPS-activated B cells, inhibits LPS-mediated secretion of IgG3 and IgG2b (17,19). Furthermore, IL-4 and IFN-γ were shown to reciprocally regulate Ig isotype production: IL-4 induction of IgG1 and IgE was inhibited by IFN-γ whereas IL-4 inhibited the IFN-γ induction of IgG2a (13,17,90).

In this regard, Mosmann and Coffman demonstrated two types of $CD4^+$ T helper subsets, known as Th1 and Th2, which segregate according to IFN-γ vs IL-4 production, respectively (91). More generally, the Th1 subset exclusively produces, upon activation, IL-2, IFN-γ, and lymphotoxin and appears to play a key role in cell-mediated immunity against intracellular pathogens and viruses (91). In contrast, the Th2 subset exclusively produces IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 and has been shown to play a protective role in certain parasitic infections (91). This paradigm led to the demonstration that Ig class switching *in vivo* could be differentially regulated by the number and/or activation state of these two T cell subsets.

Although *in vitro* studies are important for suggesting potential pathways of immune activation, antigenic challenge of the intact host involves a far more complex interplay of stimuli. Thus, unlike conditions typically manifest *in vitro*, *in vivo* the intact organism demonstrates a complex organization of B and T lymphocytes, macrophages and dendritic cells compartmentalized into distinct regions of lymphoid tissue. Thus, B cells interact with accessory cells, cytokines and other mediators, and extracellular matrix components

in a particular spatio-temporal fashion which ultimately affects the final outcome of their functional response after *in vivo* antigenic challenge (92). For these reasons it is necessary to use *in vivo* systems, in order to determine the relative importance and physiologic context of observations made on cytokine regulation of Ig class switching *in vitro*.

Such *in vivo* studies have indeed demonstrated a critical role for IL-4 in the generation and maintenance of essentially all polyclonal and antigen-specific IgE responses studied in mice (10,11,89,93,94). However, both IL-4-dependent and independent pathways of IgG1 production have been revealed, dependent upon the nature of the antigenic challenge (10,11,89,95,96). Similarly, IFN-γ-dependent and independent pathways for IgG2a secretion *in vivo* have been demonstrated (85). A reciprocal regulation of Ig isotype production by IL-4 and IFN-γ during *in vivo* immune responses has also been demonstrated (11,85,89).

The pattern of Ig isotypes produced during an immune response depends not only on the cytokines produced but also on the nature of the B cell activator. Various types of B cell activators have been used in the *in vitro* analysis of isotype switching including: LPS, mIg crosslinkers, T cells, T cell membranes, and CD40 ligand (CD40L). The B cell activator, LPS, is purified from the cell walls of gram negative bacteria. The lipid A portion of LPS purified from E. coli has been demonstrated to be the B cell mitogenic component (97). CD40L is an inducible T cell surface molecule that crosslinks

CD40 on the surface of B cells leading to a distinct type of B cell signaling (98-100). Such signalling, which occurs during cognate T cell-B cell interactions, has been shown to be important for the generation of TD responses *in vivo* (101) The cloning of the gene for CD40L has allowed for studies on the role of this molecule in B cell activation, independent of the potential actions of other T cell surface molecules (102-103). Multivalent antigen receptor crosslinking, utilizing a polyclonal population of B cells, has been accomplished through the use of anti-IgD or anti-IgM conjugated to dextran.

LPS induces resting B cells to enter the G1 phase of the cell cycle, proliferate, secrete large amounts of IgM, and undergo class switching to IgG3 and, to some extent, IgG2b in the absence of exogenous cytokines (1,104-106). As discussed above LPS, due to its mitogenic nature, can be used as a model for B cell activation in response to TI-1 antigens. Anti-IgD dextran ($\alpha\delta$-dex) mimics the repeating epitope nature of the polysaccharide antigen and thus can be considered a model for B cell activation by TI-2 antigens (107-109). In a similar vein, CD40L can serve as a model for TD-like B cell stimulation.

In earlier studies, haptenated polysaccharides, such as TNP-Ficoll and TNP-dextran, were used to study the *in vitro* antibody response to TI-2 antigens. However, these systems were difficult to study given the relatively few B cells present in the spleen which were specific for any given antigen (107-109). Although anti-Ig antibodies bound to an insoluble particle had been used to

costimulate polyclonal B cells to secrete Ig antibodies, these constructs did not mimic the soluble TI-2 antigens. In order to investigate the parameters that enabled TI-2 antigens to stimulate high levels of antibody production, anti-IgD was conjugated to a dextran backbone (αδ-dex) to create a polyclonal *in vitro* model for mIg-mediated TI-2 responses (107-109). Dextran-conjugated anti-Ig antibodies stimulated higher optimal levels of proliferation than that induced by unconjugated anti-Ig, and at concentrations of anti-Ig that were 1000-fold less. These lower concentrations more closely mirrored the levels of TI-2 antigens one would expect during *in vivo* immune responses. The enhanced B cell activation was attributed to the presentation of multiple repeating anti-IgD antibodies on the dextran backbone which allowed for greater cross-linking of IgD on the B cell surface. Furthermore, the low concentrations of αδ-dex sufficient for optimal B cell activation, did not lead to significant modulation of surface IgD and was thus able to mediate repetitive stimulation of the cell over a longer period of time, relative to unconjugated anti-IgD which rapidly modulates surface IgD (107-109).

In contrast to LPS, anti-Ig-dextran, while inducing proliferation of B cells, fails to stimulate Ig secretion or class switching in the absence of exogenous cytokines. Thus, resting B cells activated by αδ-dex proliferate and, upon addition of IL-5, secrete mostly IgM, and small amounts of IgG3 and IgG1 (7,63). However, in contrast to stimulation by LPS + IL-4 which co-induces IgG1 and IgE switching, αδ-dex + IL-5 in the presence of IL-4 while inducing switching to

IgG1 does not induce switching to IgE (7). This was one of the first demonstrations in the murine system that Ig isotype production is critically dependent upon the mode of B cell activation as well as the pattern of stimulating cytokines.

In order to provide compelling evidence that a given cytokine acts as an Ig isotype "switch" factor for a particular Ig isotype, one must demonstrate that the cytokine: (1) selectively enhances the secretion of the Ig isotype, (2) increases the percentage of cells expressing that Ig isotype on their surface, (3) acts on cells initially lacking expression of that Ig isotype on their membrane, and (4) induces the activation of the C_H gene encoding that Ig isotype, prior to switch rearrangement. This latter condition will be reflected by the appearance or upregulation of germline C_H transcripts for that isotype. Additional strong evidence for establishing a cytokine as a switch factor is the direct demonstration of enhanced switch rearrangement of the particular C_H gene after exposure of the B cell to that cytokine.

Demonstration that a particular cytokine functions as a switch factor for a specific Ig isotype, does not preclude the possibility that additional factors may also induce switching to that same Ig isotype. Thus, while induction of IgE is known to be solely regulated by IL-4 in the mouse, and IL-4 or IL-13 in the human, parameters other than IL-4 may also be involved in regulating the switch to IgG1 under various circumstances. This is supported by the observation of significant, though reduced, levels of IgG1 class switching in mice

made genetically deficient in IL-4 expression by the process of homologous recombination (89)

A summary of the known cytokines which induce specific Ig class switch events in the presence of specific B cell activators are shown in Appendix A, Table 11.

Molecular Mechanisms

The C_H locus of the mouse is located on chromosome 12, 3' of the rearranged V_HDJ_H gene which encodes antigen specificity, and is comprised of eight distinct C_H genes which determine Ig isotype. This locus spans approximately 200 kb with the C_H genes appearing in the following sequence: 5' μ–δ–γ3-γ1-γ2b-γ2a-ε–α 3' (see Appendix B, Figure 21). The switch from the expression of IgM and IgD to the expression of downstream isotypes involves a recombination event that juxtaposes a new C_H gene directly 3' of the productively rearranged V_HDJ_H gene. Several mechanisms have been proposed to account for this recombination event (see diagrams in Appendix C, Figures 22-24) including: 1) looping out and deletion - recombination between two sites on the same DNA segment with looping out and excision of the intervening DNA (110-115); 2) sister chromatid exchange - recombination between two sites on sister chromatids, with one chromatid, and the cell into which it segregated, acquiring the C_H genes lost by its sister (116); or 3) homologous recombination- recombination between two homologous sites on

paired chromosomes with one chromosome acquiring the C_H genes that were deleted from its homolog (117).

Recombination via looping out and deletion is thought to be the usual mechanism by which isotype switching occurs, although recombination by the other two mechanisms has also been reported to occasionally occur. In favor of looping out and deletion, circular DNA excision products have been detected in B cells undergoing class switching (110-115) Such circles were cloned and sequenced and found to contain the deleted 5' C_H genes. Additionally, in cells that have switched to isotypes downstream of Cμ, the DNA for C_H genes 5' to the expressed C_H gene is typically either absent, or present in haploid quantity (118-129). This would not be seen with unequal crossing over between homologous chromosomes. Furthermore, triploid quantities of Cμ DNA would be present if unequal sister chromatid exchange were the mechanism of isotype switching and this has not been observed.

Class switch recombination occurs in regions of tandem DNA repeats termed switch (S) regions which are located 5' to each C_H gene except Cδ (116,130-133). These regions vary both in length and in sequence, however, all switch sequences contain multiple copies of the pentameric sequences GAGCT and GGGGT (116, 131-133). The hexameric repeat (C/T)AGGTTG is also commonly present in switch region sequences adjacent to switch recombination sites in plasmacytomas and hybridomas and is thought to be important in mediating class switching (130). The switch regions for the 4 IgG

subclasses all share significant sequence homology suggesting their derivation from a common precursor gene (133). Likewise, the switch regions for μ, ε, and α share significant homology (133). Although switching can occur at multiple sites within a given switch region, there appears to be some predilection for recombination to occur at particular sites within the switch region. These sites have been observed to contain several distinct consensus sequences which bind specific proteins of potential regulatory nature.

Two mechanisms were orignially proposed to explain how cytokines selectively regulate switch recombination to specific Ig isotypes. One hypothesis proposed the existence of a family of specific switch region recombinases that mediate recombinations to specific switch regions (132). In this view, factors that regulate switching, such as cytokines, T cells and mitogens, would selectively regulate the expression of particular isotype-specific recombinases. A second hypothesis which is currently most favored proposes that a nonspecific S region recombinase complex interacts only with "accessible" S regions and hence is termed the "accessibility model" (134,135). In this model the role of specific factors which regulate class switching would be to regulate the accessibility of specific regions for recombination specifically by inducing transcriptional activation at that site.

Although no compelling evidence has been provided for the existence of specific switch recombinases, the accessibility model has gained widespread experimental support. This model was initially

proposed independently by two laboratory groups: (1) Alt and colleagues (134,135) studied an Abelson murine leukemia virus (AMuLV)-transformed pre-B cell line that spontaneously switched *in vitro* from μ to γ2b. They demonstrated that this pre-B cell line, prior to switch recombination, produced a truncated Cγ2b-specific transcript that did not hybridize to a V_H probe but did hybridize to a Cγ2b probe as well as to a probe from the germline genomic region 5' to Sγ2b, known as Iγ2b, which is deleted after switching to γ2b (134-135). This truncated transcript was termed a germline or sterile transcript and consisted of a 5' exon (Iγ2b) spliced to a complete Cγ2b gene. It is a sterile transcript in the sense that, in the absence of a V_H transcript, translation of this germline transcript does not produce a functional immunoglobulin molecule. AMuLV-transformed cell lines have also been shown to switch to γ3 and produce germline γ3 transcripts (136, 137). In both of these cell lines, LPS stimulation, which enhances switching to IgG2b and IgG3, increased the level of germline γ2b and γ3 transcripts prior to the induction of switching to these genes (136,137). (2) Stavnezer and colleagues (135) performed similar studies using the I.29 murine B cell lymphoma in which they likewise showed a correlation between the steady-state levels of certain germline C_H RNA transcripts and the propensity of those cells to switch to those Ig isotypes. In numerous subsequent studies, it has been observed that switching to a particular Ig isotype is always preceded by the appearance or upregulation of germline C_H RNA. Furthermore, several studies employing the technique of nuclear run-on analysis demonstrated that the increases in the steady-state levels of germline C_H RNA were

correlated with increases in transcriptional rate at that site, as predicted by the accessibility model (138,139).

Whether germline transcripts simply reflect transcriptional activation at a particular site or, in addition, play an active role in the class switch process is still a matter of intense interest. One hypothesis envisions that these transcripts may participate in the formation of triple-strand complexes with C_H DNA which function to stabilize the open DNA configuration (140). Furthermore, although germline transcripts do not encode a functional Ig, they may encode a regulatory protein (141,142) or the transcripts may splice directly to VDJ-Cμ RNA and hence result in a non-deletional expression of a new Ig isotype (143-146).

Factors that induce or inhibit germline transcription have been shown to regulate the frequency of switching to that isotype. This is exemplified by the cytokine, IL-4, which inhibits germline γ3 and γ2b transcription while inducing γ1 and ε germline transcripts in LPS-stimulated cells (4,5,90,136,137,147-149). This correlates with previous cellular studies which showed that IL-4 inhibits IgG3 and IgG2b secretion while inducing the production of IgG1 and IgE (13,88). The IL-4-mediated upregulation in the steady-state levels of germline γ1 RNA has been correlated with hypomethylation (150) and induction of a DNase I-hypersensitive site (151,152) near the initiation site of germline γ1 transcription. DNase I-hypersensitive sites are typically associated with regions of DNA that bind regulatory proteins that are important in transcriptional regulation

(153), and hypomethylated regions are associated with transcriptional activation (154). LPS, which has been shown to induce high levels of IgG3 secretion and lower levels of IgG2b secretion, may also regulate switching to these isotypes at the level of C_H gene accessibility. In this regard, increased steady-state levels of germline γ3 and γ2b RNA have been shown to be induced by LPS in the absence of expression of germline transcripts, or Ig secretion, for IgG1 or IgE (136,137).

In studies of the regulation of germline ε transcription, a 179 bp region of DNA, lacking TATA and CCAAT sequences, surrounding the Iε transcription initiation sites, was found to be necessary and sufficient for mediating IL-4-dependent transcription of a reporter gene when transiently transfected into a B cell lymphoma exposed to IL-4 (138). Transcriptional activation required the combined presence of LPS and IL-4. A distinct IL-4-inducible protein was demonstrated to bind at this site (155). Further, a DNA binding protein, know as BSAP, has also been identified, through electrophoretic mobility shift assays, as being constitutively present at this 179 bp region of DNA (155,156). BSAP is a product of the *Pax-5* gene which is one of a family of genes that encode transcription factors (156). BSAP may indeed play a more general role in class switching, as suggested by its binding to regions within or near germline transcript start sites for μ, γ1, ε, and possibly γ2a (155). Additionally, BSAP has been shown to bind to an enhancer that is located 3' to the Cα gene (3' α enhancer) (155). This enhancer has been implicated by Alt and colleagues as playing a critical role in

class switching to certain isotypes (157). This was based on the study of mice in which the 3' α enhancer was deleted from the genome. B cells from these knockout mice failed to switch to IgG3, IgG2b, IgG2a, and IgE, although switching to IgG1 and IgA appeared essentially intact. It has been suggested that the binding of BSAP to the 3' α enhancer may promote interactions between this enhancer and different Ig heavy chain genes although little is known about the mechanism of 3' α enhancer action.

In addition to germline C_H gene expression, there are several lines of evidence that DNA replication is necessary in order for switch recombination to occur. Inhibitors of DNA synthesis, such as thymidine, hydroxyurea, and bromodeoxyuridine, inhibit IgG but not IgM production by LPS-stimulated B cells (104). Further inhibition of DNA replication in B cells with aphidicolin has also been shown to inhibit switching (158). Sμ deletions have been observed to occur during the first S phase in LPS-stimulated B cells again suggesting a role for DNA synthesis in class switching(105). More compelling evidence for a role of DNA synthesis in class switching was provided by Stavnezer and Dunnick in which they sequenced Sα regions from several IgA^+ B cell tumors derived from a single IgM^+ clonal precursor (159,160). The demonstration of certain nucleotide changes in these switch regions, which were most likely produced only during the process of DNA synthesis, suggested that the switch had occured during active DNA replication (116, 130, 132).

Switching has not only been shown to occur between Sμ and a downstream S region, but also to occur via sequential deletional events between two downstream S regions. This mechanism was first suggested from studies conducted on a B cell tumor demonstrating that Sμ was ligated directly to S region segments of two other non-IgM non-IgD isotypes (132,133,161,162,163). Sequential μ to γ1 to ε switching in normal LPS+IL-4-activated murine B cells has recently been demonstrated (164). Such cells were shown to co-express mIgG1 and mIgE and secrete both Ig isotypes. Additional sequencing studies in B cells undergoing class switching demonstrated switch region sequences of Sμ ligated to two different Sγ sequences, Sμ ligated to an Sγ sequence and a Sε sequence, and Sμ ligated to a Sγ and a Sα sequence (110,165,166).

TRANSFORMING GROWTH FACTOR-β (TGF-β)

TGF-β is a cytokine possessing diverse biological activities. Thus, it plays a key role in development (167-170), immunoregulation (171-181), and tissue repair (182-187). TGF-β1 is the most abundant isoform and belongs to a superfamily of structurally related regulatory proteins which includes four additional isoforms (TGF-β2-TGF-β5). TGF-β1 is a 25 kD homodimer composed of two 12.5 kD subunits joined by disulfide bonds and is a highly conserved molecule, with the amino acid sequence between human and mouse differing only by one residue (168,188,189). TGF-β was originally defined by its ability to cause anchorage-

independent cell growth and changes in cell morphology of rat fibroblasts (190) however, it has subsequently been demonstrated to be a major growth inhibitor for many cell types (169,174-179,189, 191,194). TGF-β is produced by many cell and tissue types during various stages of cell differentiation including B cells, T cells, macrophages, and platelets and is typically found at sites of inflammation (182,190,195,201). TGF-β is typically secreted in a latent form (189,197,202,203) and can be converted to an active form by exposure to acidic pH and by protease digestion (204,205). The active form can then bind, with high affinity, to TGF-β receptors which are expressed constitutively on a variety of cell types and which are inducible on lymphoid cells (174,195,206).

TGF-β is referred to as a master regulator of various physiologic processes (194,207). This has been demonstrated by its critical role as an immunomodulator, in both stimulatory and inhibitory capacities, as well as by the concomitant involvement of TGF-β in early inflammatory responses, in tissue repair, and in the establishment of memory responses. The suppressive effects of TGF-β on both murine and human immune cells has been observed in many *in vitro* studies. In this regard, TGF-β inhibits the maturation of pre-B cells into B cells (208), inhibits B cell proliferation and Ig secretion (195,209-212), decreases thymocyte and T cell proliferation and cytokine production (174,213,214), inhibits the proliferation and cytotoxic activities of $CD8^+$ T lymphocytes, NK cells, and LAK cells, and decreases hydrogen peroxide production and respiratory burst activity by macrophages (193,212,213,215). TGF-β

is not inhibitory for cell activation but rather inhibits the G1 to S transition of the cell cycle and thus inhibits cell proliferation (211).

TGF-β has been implicated in the induction of oral tolerance consistent with its immunoinhibitory properties (216,217). Further, in experimental autoimmune encephalomyelitis (EAE) the secretion of TGF-β by $CD8^+$ suppressor T cells specifically abrogates the damaging effects of encephalitogenic $CD4^+$ T cells (216). The downmodulatory role of TGF-β in the inflammatory process is further emphasized by the widespread inflammatory disease, with rapid progression to death, seen in mice made genetically deficient in TGF-β expression by homologous recombination (218).

TGF-β also has immunostimulatory properties. Thus TGF-β promotes the IgM to IgA class switch in both mice and humans (23,26,27,219). Additionally, it is thought that TGF-β may indirectly stimulate Ig secretion by down-regulating $CD8^+$ suppressor T cells (220). Furthermore, TGF-β plays a critical role in inducing cell recruitment and activation during the early phases of an inflammatory response. Specifically TGF-β: 1) induces monocyte chemotaxis and the production of various cytokines including IL-1, IL-6, TNF-α as well as the autocrine production of TGF-β (221), 2) upregulates CD16 (FcγRIII) expression by monocytes (221), 3) promotes chemoattraction of T cells and neutrophils (222-223), 4) stimulates the expression of integrins and matrix-degrading enzymes (173), and 5) stimulates memory cell development by naive *in vitro* IL-2-activated T cells (224).

The multiple functions of TGF-β are mediated by several cell surface receptors. Five different TGF-β receptors (type I-V) have been identified in various cell types, however, type IV and V have not been found in lymphoid cells and have only been identified in pituitary cells and bovine liver membranes, respectively (188,225). Type I and type II receptors are transmembrane proteins that form a heteromeric complex upon binding TGF-β1 (188,226-229). The type III receptor, known as betaglycan, is also a transmembrane protein but has a relatively short cytoplasmic tail (230-231). Recent studies show it has no cytoplasmic signal-transducing domain and suggest that there may be direct physical interaction between type II and type III receptors on the cell surface allowing the type III receptor to regulate the ligand-binding ability or surface expression of the type II receptor (230-231).

The TGF-β1 signaling pathway has been extensively studied in MV1Lu cells where it has been demonstrated that TGF-β1 binding to the type I/type II receptor complex activates the serine/threonine protein kinase domain of the type II subunit (229,232). This kinase activity stimulates phospholipase C activity with subsequent breakdown of membrane inositol phospholipids to form inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 stimulates the release of calcium from intracellular calcium stores within the endoplasmic reticulum. Intracellular calcium, along with DAG, activates protein kinase C (PKC) to induce a calcium influx and to phosphorylate a number of proteins downstream in the signaling

pathway (233-235). TGF-β-mediated signaling also effects G protein-mediated induction of protein kinase A (PKA) activity (236). The signaling events that follow PKC and PKA activation are not yet understood, however, it is known that in cells for which TGF-β1 functions to enhance proliferation, retinoblastoma protein (Rb), a cell cycle regulatory factor, is phosphorylated and changes conformationally resulting in the release of transcription factors, one of which has been identified as E2F. E2F is transported into the nucleus where it can enhance or suppress transcription of *c-myc*, a nuclear oncogene whose product is thought to regulate a series of genes required for orchestrating the induction of cell proliferation (227,228,236-239). Furthermore, the transcription factor AP-1 can bind to the Rb control element and stimulate transcription of c-*jun*, *jun* B, and c-*fos*, which are additional nuclear oncongenes that function in transcriptional regulation (227,228,236-239). In cell types for which TGF-β1 inhibits cell growth, TGF-β1 has also been shown to be inhibitory for proteins termed cyclins which are indirect regulators of the cell cycle (240). Specifically, TGF-β inhibits the transcription of *cdk4*, a cyclin required for activation of cyclin-dependent protein kinase 2 (CDK2). This ultimately inhibits the formation of the CDK2-cyclin E complex which phosphorylates the Rb protein thus blocking the progression of cells into the S phase of the cell cycle by preventing activation of early genes required for cell division. Rb phosphorylation can also be inhibited directly by the tumor suppressor, p53.

INTERFERON-γ (IFN-γ)

Interferons were initially described on the basis of their anti-viral activity (241) which is mediated predominantly by two mechanisms (242,243): 1) double stranded (ds)RNA-dependent protein kinase blockage of the translation initiation complex and 2) dsRNA-induced production of 2-5A synthetase which activates IFN-induced endoribonucleases to cleave mRNAs and rRNAs. Both of these mechanisms result in inhibition of cellular as well as viral protein synthesis. The family of interferons includes three forms (α, β, γ) which typically elicit species-specific effects including inhibition of cell proliferation and modulation of immune responses in addition to its direct anti-viral activity. These three types are distinguished by their biochemical characteristics and the cell types from which they are isolated. IFN-γ, formerly called Type II IFN or immune IFN, is a glycoprotein synthesized by T lymphocytes and NK cells with little homology to IFN-α or IFN-β.

IFN-γ plays a central role in cell-mediated immunity (CMI) by enhancing the activity of macrophages, cytolytic T cells, and NK cells against infected cells (244-247). Additionally, IFN-γ has a variety of inhibitory and stimulatory effects on B cell function. IFN-γ is produced by the Th1 subset of CD4+ T lymphocytes, as well as NK cells and CD8+ T cells which have been strongly implicated in mediating CMI and delayed-type hypersensitivity responses (248), and also down-modulates several B cell activities induced by the Th2-derived cytokine IL-4. These activities include the IL-4-

induced increase in resting B cell size and B cell expression of MHC Class II (249,250), Thy-1 expression (251-253), and induction of the intermediate affinity receptor for IgE (FcεRII), as well as the IL-4-mediated enhancement of IgE production (254,255). In this manner it is thought that Th1 cells, via the release of specific cytokines such as IFN-γ, play a role in shifting immune responses from humoral immunity to CMI.

However, the role of IFN-γ in humoral immunity appears to be more complex. Thus, IFN-γ has both inhibitory and stimulatory effects on B cell maturation to Ig secretion, depending upon the manner in which the B cell has been activated. In the presence of IL-2, IFN-γ enhances the Ig plaque-forming cell (PFC) response to soluble protein antigens and to sheep RBC (256-258). Additionally IFN-γ-containing Th1 supernatant stimulates Ig secretion by small B cells activated with αδ-dex (7). In contrast, IFN-γ-containing Th1 supernatant inhibits the Ig PFC response by TNP-specific B cells, WEHI 279 cells, and LPS-activated B cells in the presence of IL-2 (259). With regard to class switching, IFN-γ inhibits the LPS-induced switch to IgG3 but stimulates IgG2a class switching by both LPS- and αδ-dex + IL-5- activated B cells (260). Furthermore, the *in vivo* role of IFN-γ in IgG2a production has been demonstrated following several distinct types of immunization. Small increases in serum IgG2a are seen in mice injected with goat anti-mouse IgD antibody (GαMδ) and can be enhanced by injection of exogenous IFN-γ (18). These increases are inhibited by injecting a neutralizing anti-IFN-γ monoclonal antibody (18). Additionally, injection of the heat-killed

bacterium *Brucella abortus*, a known inducer of IFN-γ secretion, stimulates high levels of serum IgG2a production (18).

The ability of IFN-γ to induce the expression of IgG2a, an Ig isotype shown to be predominantly produced in response to viral infections, and to down-modulate Th2 responses which would be inappropriate or inhibitory to the induction of IgG2a, suggests that this cytokine promotes a distinct humoral immune response that synergizes with CMI to allow for optimal host protection against viral agents. Our demonstration in this thesis that IFN-γ also induces IgG3 switching further supports this notion.

The signal transduction pathway of IFN-γ is not characterized by the involvement of typical second messengers such as cyclic adenosine triphosphate (cAMP) or inositol triphosphate (IP3), but rather has been shown to involve the activation of a newly discovered subgroup of tyrosine kinases known as the Janus kinases (261-270). Upon binding of IFN-γ to its cell-surface receptor, two Janus kinases (Jak1 and Jak2), which are thought to be bound to the intracytoplasmic portion of the receptor, become activated via phosphorylation on tyrosine residues (271). A model has recently been proposed by H. Johnson and B. Szente to describe how binding of IFN-γ to the extracellular portion of IFN-γ receptors activates intracellular tyrosine kinases (274). This model is based on their demonstration that when IFN-γ receptor molecules are exposed to IFN-γ, the C terminal domain of IFN-γ can then bind to an intracytoplasmic portion of the receptor. The proposed model is as

follows: After IFN-γ binds to specific cell surface receptors, the IFN-γ/IFN-γ-receptor complex is drawn into the cytoplasm by receptor-mediated endocytosis. Following internalization, IFN-γ becomes dissociated from its receptor and is then able to bind through its C terminal domain to a specific binding site on the intracytoplamic region of the IFN-γ receptor. In this manner, the Jak kinase is displaced from the receptor and can then be phosphorylated to become an active enzyme that can then act on other proteins. It has been shown that these activated enzymes then phosphorylate a 91-kD protein known as Stat91 (271). This protein, previously referred to as γ-activated factor (GAF), was shown to preexist in the cytoplasm (271), to be activated rapidly in response to IFN-γ, and to disappear within 2-3 hours (273). Stat proteins are so named because they function both in *s*ignal *t*ransduction as well as *a*ctivation of *t*ranscription. Activated Stat proteins are translocated to the nucleus where two phosphorylated Stat91 protein molecules are thought to associate with a 43-kD protein (261). Phosphorylation of Stat91 on tyrosine residues in the SH2 pocket has been shown to be necessary for Stat dimerization which is critical for the DNA-binding activity of this protein (273) and hence for responsiveness to IFN-γ (272). Together these three proteins form a complex within the cell nucleus which attaches to genes whose promoters contain an IFN-γ response element known as interferon-gamma activation site (GAS) (261,274). This is a region demonstrated to consist of a repeated 9-bp core sequence (TTNCNNNAA) comprising a unit of 20-30 bases (261). Binding of this protein complex to GAS then induces activation of transcriptional machinery with resultant production of mRNA. An

84-kD Stat protein which differs from Stat91 only in that it lacks the COOH-terminal 38 amino acids is unable to activate gene transcription through GAS elements (261). Thus it appears that this region in the carboxy-terminus of the Stat91 protein is critical for gene activation.

II. MATERIALS AND METHODS

Mice. Female DBA/2 and BALB/c mice were obtained from the National Cancer Institute (Frederick, MD) and were used at 8-12 weeks of age. The experiments were conducted according to the principles set forth in the *Guide for the Care and Use of Laboratory Animals*, Institute of Animal Resources, National Research Council, Department of Health, Education and Welfare Publication No. 78-23 (National Institutes of Health).

Culture medium. RPMI 1640 (Biofluids, Rockville, MD) supplemented with 10% FCS (Sigma Chemical Co., St. Louis, MO), L-glutamine (2mM), 2-mercaptoethanol (0.05 mM), penicillin (50 μg/ml), and streptomycin (50 μg/ml) was used for culturing cells.

Reagents.

B Cell Activators: **LPS** W, extracted from *Escherichia coli* 0111:B4, was obtained from Difco Laboratories, Inc. (Detroit, MI) and was used at 20 μg/ml in all experiments. Dextran-conjugated monoclonal anti-IgD (Hδa/1) (**αδ-dex**) was prepared by conjugation of Hδa/1 (monoclonal mouse IgG2b (b allotype), anti-mouse IgD (a allotype) to a high m.w. dextran (2 x 10^6 kd) as previously described (107). Anti-IgD antibodies were conjugated to dextran at a ratio of 9:1. The concentrations of αδ-dex noted in the text refer only to that of the anti-IgD portion of the conjugate and not to the entire dextran conjugate. Recombinant **CD8-CD40 ligand** (L) fusion protein was

purified by Lane et al. (102) from culture supernatants by precipitation with ammonium sulfate and chromatography over a CM-Sepharose column as previously described.

Cytokines: Purified porcine **TGF-β1** was obtained from R&D Systems, Inc. (Minneapolis, MN). Murine **rIL-5**, prepared in the baculovirus system, was a kind gift from Dr. Richard Hodes (National Institutes of Health, Bethesda, MD). Murine **rIL-4**, produced in *E. coli*, was a generous gift from Dr. A. D. Levine (Monsanto Corporate Research, St. Louis, MO). Murine **rIFN-γ**, prepared from Chinese hamster ovary cells, was a kind gift of Genentech (South San Francisco, CA). Murine **rIL-2** was the kind gift from Dr. Maurice Gately (Hoffman-La Roche, Nutley, NJ). **rIL-10** was purchased from Pharmingen.

Anti-cytokine antibodies: The following mAb's were purified from ascites: Mouse IgG1 **anti-TGF-β** mAb (1Dll.16.8) neutralizes both TGF-β1 and TGF-β2 and was a generous gift from Celtrix Pharmaceuticals, Santa Clara, CA. Mouse IgG1 anti-mouse $IgM^{b\text{-allotype}}$ (**MB86**) mAb was used as an isotype-matched control for 1D11.16.8. **XMG-6** is a rat IgG1 anti-mouse IFN-γ mAb. **J4-1** is a rat IgG1 anti-NP mAb and was used as an isotype-matched control for XMG-6. MB86, XMG-6, and J4-1 mAb's were obtained through the generous contribution of Dr. Fred Finkelman, USUHS, Dept of Medicine.

CD4+ T cell clones: D10.G4.1 (D10) (conalbumin-specific, IaK-restricted) was considered a Th2 clone based on its secretion, after stimulation, of IL-4 but not IFN-γ or IL-2 (275). **A.E7** (cytochrome c-specific, Ia^{k}-restricted) was considered a Th1 clone

based on its secretion, after activation, of IFN-γ and IL-2, but not IL-4 (276). Cytokine-rich supernatants were previously prepared as follows: Resting D10 (1 x 10^5/ml) or A.E7 (1 x 10^6/ml) T cells were stimulated by adding irradiated (3000 R) spleen cells (5 x 10^5/ml (D10) or 5 x 10^6/ml (A.E7)) from C3H mice (Ia^k-bearing), acting as APC, in the presence of 100 μg/ml of conalbumin or cytochrome c (Sigma, St. Louis, MO), respectively. Cellfree supernatants were harvested 25h later, aliquoted, and stored at -80°C.

cDNA probes: The **Iγ1** cDNA probe (a kind gift from Dr.W.A. Dunnick, Univ. of Michigan, Ann Arbor, MI) was a 2 kb BamH1 fragment located at the I region 5' to Sγ1 and therefore was specific for germline γ1 RNA only. This probe binds to both the membrane and secretory forms of the germline γ1 RNA transcript (277-279). The **Iγ2b-Cγ2b** cDNA probe was used in the S1 nuclease protection assay and was generated previously as described below. The **Iγ3 cDNA** was a 600 bp EcoRI/AccI fragment from the Iγ3 region and therefore was specific for germline γ3 RNA (136).

Staining reagents for flow cytometric analysis: FITC-labelled monoclonal rat IgG2a anti-mouse IgG2b, FITC-labelled monoclonal rat IgG2a anti-mouse IgG3, FITC-labelled monoclonal rat IgG1 anti-mouse IgA were purchased from Pharmingen, San Diego, CA. FITC-labelled monoclonal rat IgG1 anti-mouse IgG1 was purchased from Zymed Laboratories, South San Francisco, CA. Phycoerythrin-labelled, affinity purified polyclonal goat anti-mouse IgM was purchased from Southern Biotechnology Associates, Birmingham, AL. Rat IgG2b anti-mouse FcγRII (2.4G2) was purified from ascites.

ELISA antibodies: Unlabeled and alkaline phosphatase-conjugated polyclonal goat anti-mouse Ig isotype specific antibodies for ELISA were obtained from Fisher Scientific, Pittsburg, PA. Rat IgG2a anti-mouse IgE (EM95), rat IgG1 anti-mouse IgE (R1E4), and rabbit anti-mouse IgE (EMG5) were purified from ascites.

Preparation of resting B cells by discontinuous Percoll gradient centrifugation. Enriched populations of B lymphocytes were prepared from spleen by eliminating T lymphocytes in the following manner. Mice were sacrificed by cervical dislocation. The spleen was removed and placed in a sterile 100mm culture dish containing 5-10 ml of cold, red Hank's Balanced Salt Solution (HBSS) (Biowhittaker, Walkersville, MD) containing 1% (50 mg/ml) penicillin/ streptomycin (Biowhittaker). Using the flat end of a sterile 10 ml syringe, the spleens were disrupted and cells were released into the medium. The HBSS-cell suspension was pipetted into a 50 ml conical tube, brought to a total volume of 50 ml, and centrifuged at 1000 rpm for 10 minutes. Following centrifugation, the supernatant was discarded and the cell pellet was suspended in a sterile filtered anti-T cell antigen cocktail containing anti-Thy 1.2 ascites, rat IgG2b anti-CD4 (clone GK1.5), rat IgG2b anti-CD8 (clone 2.43), and HBSS. Following incubation on ice for 30 minutes, the cell suspension was brought to a total volume of 50 ml with HBSS and centrifuged at 1000 rpm for 10 minutes at 4°C. The supernatant was dicarded and the cell pellet was resuspended in a sterile filtered complement cocktail containing guinea pig complement (GibcoBRL,

Long Island, NY), (prewarmed to 37°C), mouse anti-rat Igκ (clone MAR 18.5), hepes (Biowhittaker), and HBSS. Following incubation at 37°C for 30 minutes, the cell suspension was brought to a total volume of 50 ml with HBSS and centrifuged at 1000 rpm for 10 minutes at 4°C. The supernatant was discarded and the cells were resuspended in a total volume such that 2 ml of HBSS contained cells from 1-3 spleens. Cells were then fractionated into low and high density populations by discontinuous Percoll (Pharmacia, Piscataway, NJ) gradient centrifugation. 50%, 60%, 66%, and 70% percoll solutions were prepared by dilution of 100% percoll with either HBSS or RPMI 1640 (Biowhittaker). (For visualization of percoll bands, the diluent was alternated for every other percoll concentration.) The percoll gradient was formed in a 15 ml conical tube by slowly pipetting first the 70% percoll solution, followed by the 66%, 60%, and 50% percoll solutions. The gradients were chilled on ice for 15 minutes followed by layering 2 ml of cell suspension over the upper (50%) percoll layer and 20 minute centrifugation at 3000 rpm, 4°C. After centrifugation, the cells which banded at/or between the various percoll densities are as follows: above 50% - dead cells, some macrophages, and large activated B cells; 50-60% - macrophages, activated B cells; 60-66% and 66-70% - small resting B cells; below 70% - small B cells and RBC's. For the studies included in this thesis, cells that formed a band between 66 and 70% solutions (with densities between 1.086 and 1.081 g/ml) were harvested. This population of resting, high density, T-depleted spleen cells consisted of approximately 90% B cells as measured by flow cytometric analysis of B220+ (mAb 6B2) cells. The cells from this layer were

then washed by resuspending in a total volume of 15 ml HBSS and centrifuged at 1000 rpm for 10 minutes at 4°C. The supernatant was discarded and the cells were adjusted to a stock concentration of 1 x 10^7 cells/ml by the addition of supplemented RPMI 1640. Experiments using cell preparations were set up either on the day of cell preparation or the following day.

B cell cultures. Functional assays were carried out in 96- and 24-well flat bottomed Costar plates (Costar, Cambridge, MA). Cells were typically cultured at a final concentration of 1.25 x 10^5 cells/ml (unless otherwise noted) and incubated at 37°C in a humidified atmosphere containing 6% CO_2.

Ig isotype-specific ELISA for quantitation of secreted Ig. Ig isotype concentrations of supernatants harvested on day 6 of culture were measured by direct ELISA. Immulon 2, 96-well flat-bottomed ELISA plates (Dynatech Laboratories, Inc., Alexandria, VA) were coated with 10 µg/ml polyclonal goat anti-mouse Ig of the specific isotype being analyzed (50 µl/well). These antibodies were obtained from either Southern Biotechnology, Birmingham, AL or Fisher Biotech, Pittsburg, PA except for analysis of secreted IgE. The coating antibody used for IgE studies was the rat monoclonal anti-IgE, EM95, (15 µg/ml) obtained by the generous contribution from Dr. Fred Finkelman, USUHS, Department of Medicine. Dilutions of coating antibody were made in borate buffered saline (BBS), 50 µl was added to each well of a 96-well plate, and plates were typically incubated overnight at 4°C. Plates were washed three times with

distilled water to remove unbound coating antibody. Protein carrier solution (PCS) (50 µl) containing BBS, 1% fetal calf serum, and 0.1% sodium azide, was added to each well to block sites of microtiter wells where coating antibody may not have bound. After a 30 minute incubation at room temperature, plates were washed three times with distilled water. Various dilutions of culture supernatants (1/1, 1/5, 1/25, 1/125, 1/625) and serial 1:2 dilutions of Ig isotype standards (myeloma proteins of known concentrations) were made in PCS and added to appropriate microtiter wells. Plates were incubated for two hours at room temperature followed by two sets of three distilled water washes separated by the addition of BBS-Tween (50 µl/well) for ten minutes. These washes were performed to remove unbound antibody prior to the addition of alkaline phosphatase (AP)-conjugated anti-isotype antibody (Southern Biotechnology, Birmingham, AL) (50 µl/well) which was diluted with PCS to the appropriate concentration (typically 100 ng/ml for all isotypes analyzed). Following a two hour incubation at room temperature, the plates were washed three times with distilled water, once with BBS-Tween (50 µl/well) for ten minutes, and three additional times with distilled water. After discarding all water in the wells, 4-methyl umbelliferyl phosphate (MUP)(Sigma Immunochemicals, St. Louis, MO) substrate solution (50 µl/well of a 0.05 mg/ml solution) was added to each well. A fluorescent product was generated by cleavage of MUP by the specifically bound AP-conjugated antibodies. Ig isotype concentrations were determined on a 3M FluoroFAST 96 fluorometer (Becton Dickinson, Mountain View, CA) and fluorescence units were converted to Ig concentration

(ng/ml) by interpolation from standard curves. Each assay system had been previously assessed to have no cross-reactivity or interference from other Ig isotypes found in the culture supernatants.

ELISPOT Assay. Quantitation of specific Ig-isotype secreting cells was performed by this protocol and was done in collaboration with Dr. Dennis Klinman, FDA, Bethesda, MD. (281) Cultured cells were harvested 4.5d after initiation of culture and washed in fresh medium to eliminate residual Ig secreted by cells in culture medium. Flat-bottom, Immulon 1 microtiter plates (Dynatech Laboratories Inc., Chantilly, VA) were coated with goat anti-mouse Ig and blocked with 1% BSA in PBS. Serial dilutions of cell suspensions were made in supplemented RPMI, with the initial cell concentration being 2 x 10^5 cells/ml in 200 µl, and added to anti-Ig coated plates. Plates were centrifuged at 500 rpm for 3 minutes then incubated for 7 h at 37°C in a 5% CO_2 incubator. Following incubation, the cells were washed away with three PBS/0.05% Tween 20 washes and three distilled water washes. After washing, AP-conjugated anti-mouse Ig isotype-specific antibodies (Southern Biotechnology) were added to each well and the plates were incubated for two hours at room temperature. After incubation, the washing steps were repeated as before followed by overlaying each well with agarose (Sigma, Type I-low melt, 0.03 gm/ml) prepared in a 5-bromo-3-chloroindolyl phosphate-containing solution (Sigma Immunochemicals) and kept at 50°C. Ig produced by individual B cells bound to the plate were visualized when the alkaline phosphatase specifically bound to these

plate-adhered B cells cleaves 5-bromo-3-chloroindolyl phosphate to produce a blue spot in the agarose overlay. No diffusion occurs once the agarose solidifies at room temperature. The dilution of cells producing 20-40 blue spots/well was used to determine the total number of Ig-specific B cells/sample.

LPS/anti-TGF-β immunization protocol. For *in vivo* experiments, five mice per group were analyzed. Mice were bled, either intraocularly or by tail vein incision, 3-7 days prior to immunization. These prebleed serum samples were used as control samples in that they typically contained low concentrations of LPS-specific Ig. These low results were expected since it was anticipated that these mice had no previous exposure to this antigen. Thus, this analysis also served as an indicator of potential previous or current infection with LPS-containing microorganisms.

For mice receiving LPS only, mice were immunized by tail vein injection with the appropriate concentration of LPS (described for each experiment) contained in a volume of 0.1 ml. A control group was immunized with Hank's Balanced Salt Solution (HBSS) at the same time LPS immunizations were performed. Serum samples were obtained on the 6th and 9th day following immunization in the manner previously mentioned. All serum samples were kept frozen until they were analyzed simultaneously by LPS-specific ELISA. HBSS-injected mice typically generated similar LPS-specific Ig levels similar to those observed for prebleed samples.

For experiments in which mice were immunized with anti-TGF-β mAb (1Dll.16.18) (280) and LPS, prebleeds were performed as

described. Mice were then immunized with the first dose of antibody one day prior to LPS immunization followed by a second dose on the fourth day after LPS immunization. 2 mg of anti-TGF-β mAb in a total volume of 0.1 ml was administered per immunization. Serum samples were then obtained on the 6th and 9th day following LPS immunization.

LPS-specific ELISA. This assay was performed to analyze the titers (or concentration) of LPS-specific Ig isotypes present in serum following LPS immunization of mice. 96-well, U-bottom microtiter plates (Dynatech Laboratories Inc., Chantilly, VA, catalog no. 001-010-2201) were coated with LPS (25 μg/ml diluted in sodium carbonate coating buffer; 50 μl/well) at 4°C for 2-3 days. Following incubation, plates were washed three times with BBS-Tween. Samples were diluted in BBS-Tween and 50 ml of each sample was added to appropriate microtiter wells. (When analyzing titer point, a 1:50 initial dilution of serum was made followed by serial 1:2 dilutions across the microtiter plate. After obtaining LPS-specific, isotype-specific antibodies of known concentration for use as standards, the assessment of Ig concentration was possible, and samples were diluted as described above for conventional ELISA.) Following a 30 minute, room temperature incubation, the plates were washed three times with BBS-Tween prior to the addition of secondary AP-conjugated anti-Ig specific antibodies (50 μl/well; 200 ng/ml). The plates were incubated at room temperature for one hour followed by three BBS-Tween washes and the addition of MUP substrate solution (100 μl/well). Fluorometric readings were

performed as described for conventional ELISA. When LPS-specific Ig isotype standards were used, fluorometric analysis was performed as desribed for conventional ELISA. The comparative evaluation of LPS-specific Ig isotype titers was made as follows: The average fluorescence unit obtained for serum samples from all mice prior to LPS immunization (prebleeds) was defined as the titer point for that specific experiment. Serum samples for an entire experiment, including prebleeds and post-LPS immunization bleeds, were analyzed simultaneously. For each serum sample analyzed after LPS immunization, the fluorescence units, and their respective serum dilutions, which bracketed the defined titer point were used to calculate by linear regression the actual dilution of the serum sample which equalled the titer point. The reciprocal of this titer was then reported and used for comparative analysis between various serum samples. (The greater the dilution required to equal the titer point, the higher the level of LPS-specific Ig.)

Flow cytometric analysis. B cells, harvested on the fourth day of culture, were washed in HBSS then incubated for 20 min with 5 μg/ml of rat IgG2b anti-FcγRII mAb (2.4G2) to prevent cytophilic antibody binding. This was followed by staining 100 μl of 10^6-10^7 cells/ml for 30 minutes with various combinations of fluorochrome-labelled immunoreagents (final concentration of 10 μg/ml) in cold clear HBSS containing 3% FCS. Cells were then washed and resuspended in 300 μl staining buffer (final cell concentration approximately 10^5-10^6 cells/ml. All samples were kept on ice throughout staining and analysis. Fluorescence analysis was

accomplished on a Becton Dickinson FACStar Plus (Mountain View, CA) set for logarithmic amplification. 15,000 cells were typically collected and analyzed. Viable lymphocytes were identified on the basis of their characteristic forward and side scatter profiles and their exclusion of propidium iodide (Sigma).

Fluorescence activated cell sorting (FACS). Cells were prepared and stained in the manner described above for flow cytometric analysis with the exception that cells were typically brought to a final concentration of 10^7 cells/ml and, depending on the assay, 3-6 x 10^7 cells were provided for sorting. The conditions for individual sorting experiments are described within the text and figure legends. Electronic cell sorting was performed on an EPICS Elite cytometer (Coulter Corp., Hialeah, FL). Sorted cells were reanalyzed immediately upon their isolation to assess the level of cell purity. This equals the percentage of cells of the desired cell population and is described for individual experiments.

Measurement of B cell proliferation and assessment of viable cell yields. DNA synthesis was determined by [^{3}H]TdR uptake by pulsing the culture wells with [^{3}H]-TdR 48 hours after initiation of culture over a 4-hr period (2μCi/well; 6.7 Ci/nmol; 1mCi = 37 GBq; ICN, Irvine, CA). Cells were harvested (PHD cell harvester, Cambridge Technology, Watertown, MA) onto glass fiber filters and [^{3}H]TdR incorporation was determined by liquid scintillation spectrometry. The results obtained are expressed as counts per minute.

Viable cell yields were determined on the fourth day after initiation of culture. The cells in the culture wells were resuspended by pipetting and a 100 µl aliquot was removed and mixed with an equal volume of the vital dye, trypan blue. A 10 µl aliquot was placed in a hemocytometer counting chamber and the viable cells were identified on the basis of dye-exclusion.

RNA preparation. Total RNA was extracted from cells for the analysis of germline C_H Ig isotype-specific RNA transcript production by Northern blot analysis using the RNAzol B method (Tel Test, Inc., Friendswood, TX). Cells were harvested 3 d after initiation of culture, the number of cells present in culture was determined and cell suspensions were centrifuged at 1000 rpm for 10 minutes. The medium was decanted and the cells were lysed by the addition of 0.2 ml of RNAzol B per 10^6 cells. The lysate was mixed several times with a pipette to ensure solubilization of the RNA and 0.2 ml of chloroform was added per 2 ml of cell homogenate to extract the RNA. The samples were mixed vigorously for approximately 15 seconds then placed on ice for 5 minutes. Following incubation, the tubes were centrifuged at 12,000 g for 15 minutes at 4°C. After centrifugation, the upper aqueous phase containing the RNA was removed and placed in a fresh tube. An equal volume of isopropanol was added and the samples were incubated on ice for 15 minutes, followed by centrifugation at 12,000 g for 15 minutes, 4°C. The supernatant was decanted and the RNA pellet was washed with 75% ethanol (0.8 ml/50-100 µg RNA) by vortexing followed by centrifugation for 8 minutes at 7500 g, 4°C. The tubes were inverted

and allowed to air dry at room temperature for approximately 15 minutes then pellets were resuspended in 1mM EDTA, pH 7, 50 ml. A 1:100 dilution of 5 µl of each sample was made for determination of RNA concentration by UV absorption spectrophotometry at a wavelength of 260 nm. The final RNA concentration (µg/µl) was calculated by multiplying the OD_{260} by the dilution factor and by the RNA extinction coefficient (1 OD=40 µg/ml RNA) and dividing by 1000. Each sample was assessed for purity by analyzing the $O.D._{260}/O.D._{280}$ ratio. Only samples with a ratio of 1.8-1.9 were analyzed by Northern blot.

Northern blot analysis. This technique was utilized to assay for steady-state levels of germline C_H Ig-specific RNA transcripts. Samples were first prepared for formaldehyde gel electrophoresis by adding 20 µg of RNA to a cocktail containing 10X MOPS (3-N-morpholino propanesulfonic acid), formamide, and formaldehyde which results in denaturation of the RNA when heated to 55°C for 15 minutes. This was followed by quenching on ice. RNA loading buffer (3 µl) containing 50% glycerol, 1mM EDTA, 0.4% bromphenol blue, and 0.4% xylene cyanol was added to each sample and samples were loaded into wells of a 1% agarose gel which contained formaldehyde (2%) and ethidium bromide. Gel was electrophoresed at 120V for approximately 4 hours in 1X MOPS running buffer until the bromphenol blue dye front migrated approximately three-fourths down the gel.

After electrophoresis, the RNA contained in the agarose gel was transferred to nylon membrane (Micron Separations, Inc., Westboro,

MA) by capillary action. This was accomplished by establishing a salt gradient with the gel being soaked in 20X SSC then overlaid with the nylon membrane soaked in distilled water and successive layers of blotting papers soaked in 10X SSC and 5X SSC. The transfer set-up was left in place overnight. The following day, the membrane was subjected to UV-crosslinking to bind the RNA to the membrane then placed in prehybridization solution containing formamide, Denhardt's solution, 20X SSC, salmon-sperm DNA, and 10% SDS for 2 h at 40°C.

Next, the germline C_H cDNA probes (specific for Iγ3 and Iγ1) were prepared by random hexamer labelling with incorporation of ^{32}P-deoxycytidine. Unlabelled nucleotides were removed by passage of the cDNA mixture over an Elutip-d adsorption column (Schleicher and Schuell, Keene, NH) primed with a low salt solution followed by elution with a high salt solution. The level of radiolabelling of the probe was determined by liquid scintillation counting (cpm) and 10^6 cpm of denatured probe was added per ml of hybridization mixture.

Following probe preparation, the membrane containing the RNA was removed from the prehybridization solution and added to approximately 10 ml of hybridization buffer containing formamide, 20X SSC, 2M Tris, 100X Denhardt's solution, salmon sperm DNA, and the radiolabelled cDNA probe. Hybridization was performed overnight at 40°C after which time the membrane was washed three times at room temperature in a 2X SSC/10% SDS solution followed by three washes in a 0.1X SSC/10% SDS solution for 20 minutes each at 50°C. The excess solution was blotted from the membrane surface and the membrane was covered in saran wrap, placed in an X-ray

film cassette for autoradiography at -70°C and developed after 2 d exposure.

Ethidium bromide staining of 28S rRNA bands on the formaldehyde gel have been included to demonstrate that nearly equal amounts of RNA were loaded for each sample tested.

S1 nuclease protection assay. This technique provides a sensitive and stringent method for assaying steady-state levels of germline $C_H\gamma 2b$ RNA (137) and was done in collaboration with Dr. Paul Rothman, Department of Medicine, Columbia University. For generation of the probe used in this analysis, the 5' end of germ line γ2b cDNA (from the EcoRI site at the 5' end of the Iγ2b region to the BamHI site in the C_H1 domain of Cγ2b) was subcloned into M13 bacteriophage. An α-^{32}P-dATP-labelled probe complementary to the RNA sequence of interest was generated by primer extension. Hybridization of this probe to RNA containing the homologous germline transcript completely protects the Iγ2b-Cγ2b portion of the probe resulting in a 162 bp S1 nuclease-protected fragment. In contrast, hybridization of the probe to transcripts in which the Iγ2b region has been deleted (VDJ-Cγ2b) protects only the Cγ2b portion of the probe and yields a smaller (138 bp) protected fragment. This analysis was performed on a denaturing polyacrylamide gel, the gel was dried and autoradiography was performed.

RESULTS

III. A CELLULAR MODEL FOR HIGH-RATE INDUCTION OF IgA CLASS SWITCHING

INTRODUCTION

One major obstacle to understanding the mechanisms which underlie physiologic class switching to IgA has been the inability to establish an *in vitro* system for high-rate IgA class switching. TGF-β has been established as a switch factor for the murine IgA class on the basis of its ability to selectively stimulate germline α RNA and induce large increases in IgA secretion by LPS-activated cells, especially when acting in concert with IL-2 or IL-5 (23,26,282). However, IgA secretion under these conditions was associated with the generation of only a few percent mIgA$^+$ cells (25,27). This implied a requirement for additional stimuli to account for the substantially higher percentages of mIgA$^+$ cells found in Peyer's patches (10-15% of all B cells) (28), the primary site for IgA class switching *in vivo*. To address this issue, Ehrhardt et al. (27), measured the percentages of mIgA$^+$ cells generated in response to the combined action of TGF-β with various T-independent (TI) and T-dependent (TD) modes of B cell activation. The B cell mitogen, LPS, represented TI-1 activation and αδ-dex was used to activate B cells via a TI-2 pathway. Both cognate and noncognate modes of TD B cell activation were also analyzed. Independent of the nature of the B cell activator used, only 1-2% mIgA$^+$ cells were generated, even in the presence of exogenous IL-2, IL-4, IL-5, and/or a supernatant from an activated CD4$^+$ Th2 clone. In this study, however, B cell

activators were used individually and not in combination. Additionally, the ability for costimulation by a purified T cell surface molecule, such as CD40 ligand (CD40L) in combination with LPS or αδ-dex, to induce high-rate IgA class switching was not studied.

CD40 is a cell membrane glycoprotein related to receptors for TNF-α and is expressed on pre-B cells in the bone marrow, mature B cells, bone-marrow-derived and follicular dendritic cells, activated macrophages, and thymic epithelial cells (283,284). The ligand for CD40, CD40L, is a member of the TNF family of transmembrane glycoproteins and is expressed on activated T cells (284). Recently, a soluble form of CD40L was constructed by making a fusion protein consisting of the mouse CD8α chain and the extracellular region of mouse CD40L (102). Crosslinking of CD40 on the B cell surface with this, and other CD40 crosslinking reagents have been shown to promote B cell proliferation and Ig class switching (285). When CD40-CD40L interactions are blocked, B cells are unable to proliferate or produce Ig in response to T cells (285).

Several considerations impacted on our efforts to establish a high-rate IgA class switching system. First, since cell proliferation had been implicated as a key parameter for induction of Ig class switching (160) it was possible that the inability of TGF-β to induce high-rate IgA class switching could be based in part on its strong anti-proliferative effect on B cells (195). A synergistic augmentation of B cell proliferation in response to the combined effects of either LPS- (286) or CD40-mediated signalling with antigen receptor

crosslinking (283) has been observed. Thus, we reasoned that the use of synergistic growth signals in combination with high doses of TGF-β might allow for a high degree of IgA class switching by optimizing the IgA inductive effects of TGF-β while still allowing a vigorous proliferative response to occur.

As mentioned, various cytokines, including IL-2, IL-4 and IL-5, have been implicated in obtaining enhanced IgA secretion. Earlier work by Kawanishi et al. (287,288) demonstrated the importance for IL-4 and IL-5 in obtaining high levels of IgA secretion when Peyer's patch T cells were cultured with LPS-activated Peyer's patch or splenic B cells. In later studies, Sonoda et al. (289) noted a role for IL-5 in promoting the survival and maturation of $mIgA^+$ cells into IgA secreting cells. These studies, however, only demonstrated an effect of IL-4 and IL-5 on IgA secretion but no direct effect on IgA class switching. Furthermore, the effects of IL-2 or IL-5 as a B cell differentiation factor are not selective for IgA secretion.

Recent studies from our laboratory, however, indicated a novel role for IL-5 in inducing a critical component of the Ig switch recombination machinery in normal murine B cells activated through multivalent antigen receptor crosslinking using αδ-dex (290). Additionally, studies from Warren Strober's laboratory demonstrated that IL-4 could stimulate the enhanced generation of $mIgA^+$ cells by the B cell tumor, CH12.LX, which spontaneously differentiates into $mIgA^+$ cells at a low rate (291), although similar data had not been reported for normal B cells. Hence, towards the goal of establishing

an *in vitro* system for high-rate IgA class switching, initial studies focused on testing TGF-β with various combinations of B cell activators, IL-4, and/or IL-5. Subsequently, preliminary studies analyzed the ability for two additional cytokines, IL-2 and IL-10, to induce high rate IgA class switching in the presence of TGF-β and dual B cell activation. The effects of IL-2 in this system were of interest due to previous reports linking this cytokine to enhanced IgA secretion by LPS-stimulated B cells (26). The effects of IL-10 were of equal interest since IL-10 and TGF-β had been shown to cooperatively induce anti-CD40-activated naive human B cells to secrete IgA (292). Further implication for IL-10 in IgA class switching had been demonstrated by the enhanced development of chronic enterocolitis in mice genetically deficient for IL-10 (293).

RESULTS AND DISCUSSION

Multivalent antigen receptor cross-linking of CD40L- or LPS-activated B cells, plus IL-4, IL-5, and TGF-β induces a large percentage of mIgA+ cells. Initial studies were aimed at inducing high-rate IgA class switching *in vitro* by combining several distinct modes of B cell activation with a number of cytokines known to be active in regulating class switching. Thus, flow cytometric analysis of membrane (m)IgA$^+$ cells was performed to assess IgA class switching in response to activation with dual combinations of the multivalent antigen receptor crosslinker, αδ-dex, CD40L, and/or LPS, plus IL-4 + IL-5, in the presence or absence of TGF-β (Figure 1). In the absence of TGF-β, no significant induction of mIgA$^+$ cells was

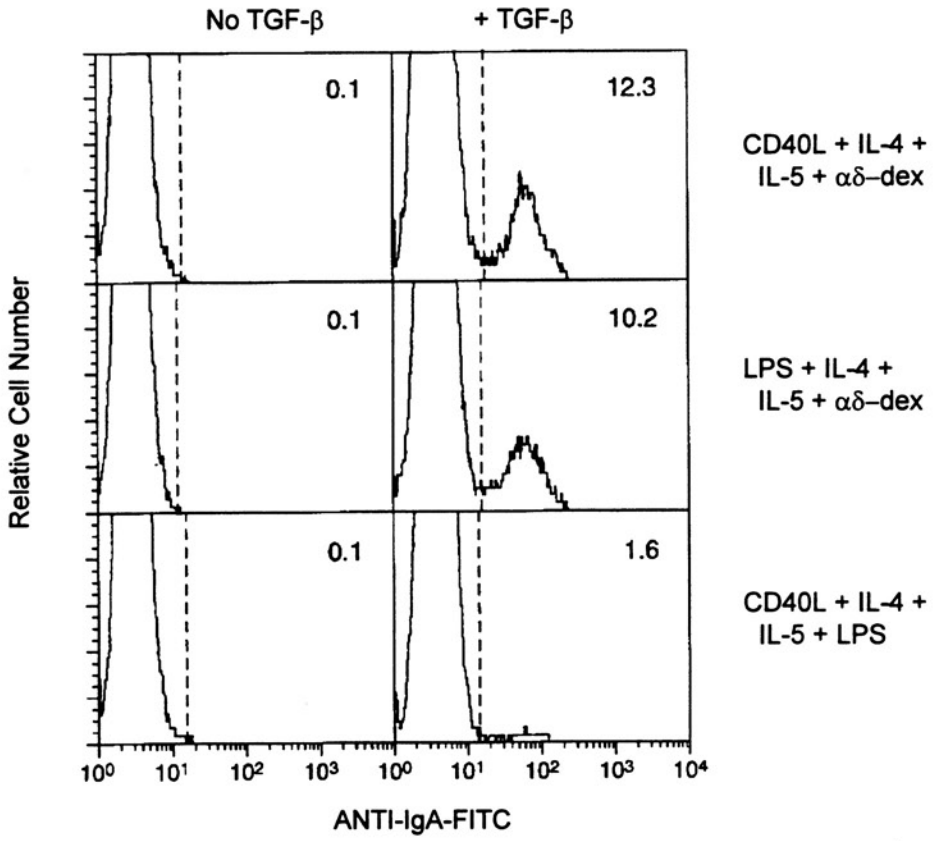


FIGURE 1. High-rate induction of mIgA+ cells upon activation with LPS or CD40L in the presence of αδ-dex, IL-4, IL-5, and TGF-β. Resting splenic B cells were activated with LPS (20 μg/ml) or CD40L (20 μg/ml) in the presence or absence of αδ-dex (3 μg/ml for LPS and 0.3 μg/ml for CD40L), IL-4 (10,000 U/ml), and IL-5 (150 U/ml), with or without TGF-β (10 ng/ml). Cells were harvested 4 days after initiation of culture for quantitation of mIgA+ B cells by flow cytometric analysis. 15,000 cells from each group were analyzed. The percentage of mIgA+ cells for each sample is shown in the upper right hand corner. Data is representative of multiple experiments.

seen, regardless of the method of activation. In the presence of TGF-β, activation with αδ-dex + IL-4 + IL-5 in combination with either LPS or CD40L generated 10% and 12% mIgA$^+$ cells, respectively. This represented greater than a 100-fold increase in the percentage of mIgA$^+$ cells over that seen in the absence of TGF-β. In contrast to costimulation in the presence of αδ-dex, dual activation with CD40L + LPS resulted in comparatively low levels of mIgA$^+$ cells.

Dose response studies demonstrated that the following concentrations of stimuli were sufficient for the generation of optimal percentages of mIgA$^+$ cells: 3 ng/ml of αδ-dex for LPS/αδ-dex costimulation, 0.3 ng/ml of αδ-dex for costimulation by CD40L/αδ-dex, 10 ng/ml of TGF-β (Figure 2), 10 μg/ml of CD40L, 10,000 U/ml of IL-4, and 150 U/ml of IL-5 (Table 1). TGF-β was selective for induction of mIgA$^+$ cells following costimulation with LPS, αδ-dex, IL-4, and IL-5 in that TGF-β did not induce mIgG2b$^+$ or mIgG3$^+$ cells nor did it affect the percentage of mIgG1$^+$ cells induced in the presence of IL-4, a known switch factor for this isotype (Table 2). Although we previously reported that TGF-β induced IgG2b class switching in LPS-activated cells (281), the presence of IL-4 in this system may have suppressed the expression of this IgG subclass (1).

Optimal induction of IgA class switching by either LPS- or CD40L-activated cells requires the independent actions of αδ-dex, IL-4, IL-5, and TGF-β. In order to determine the relative requirements of the various stimuli for generating optimal IgA class switching, we measured the percentages of mIgA$^+$

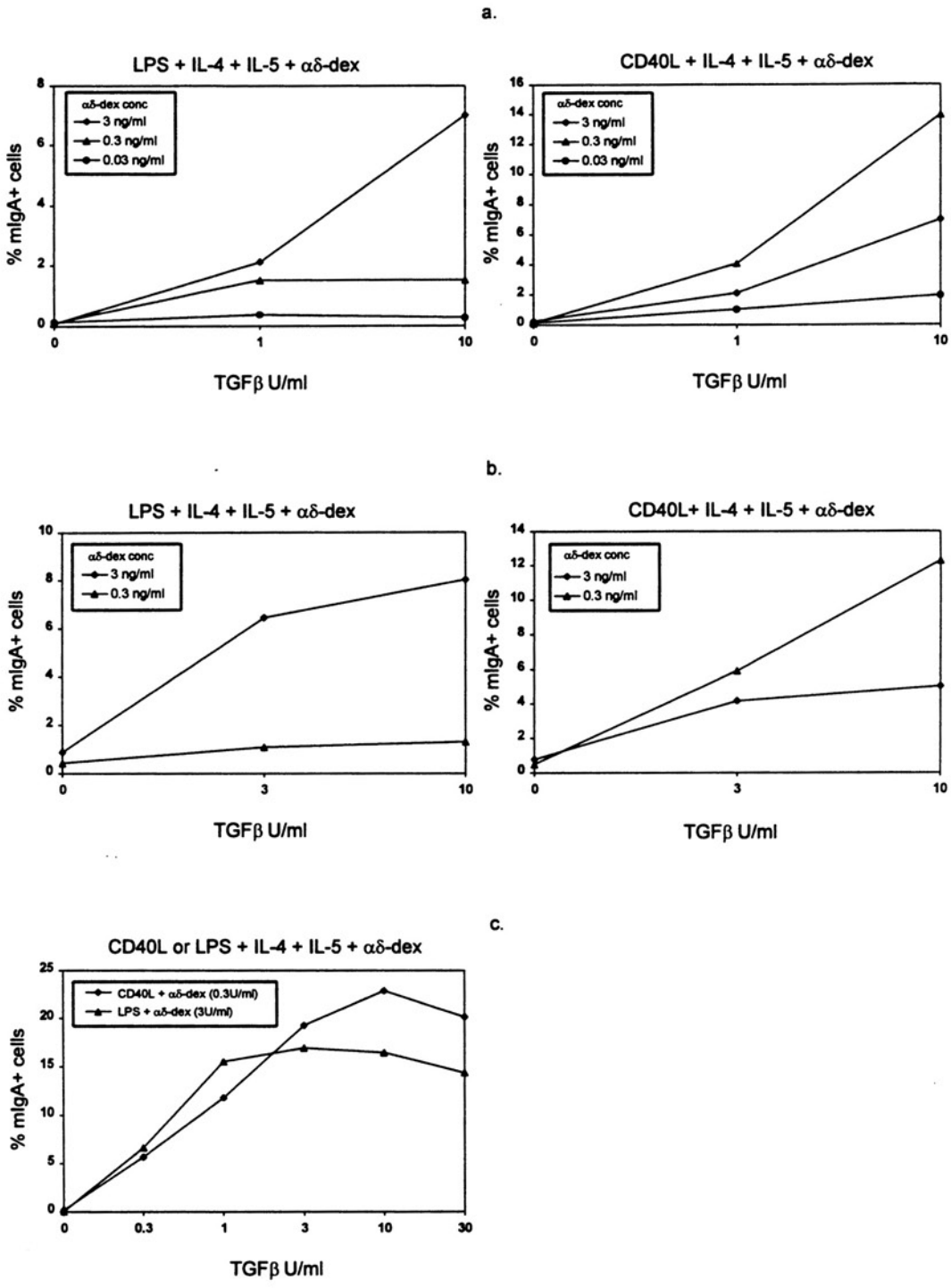


FIGURE 2. Determination of optimal concentrations of αδ-dex and TGF-β for inducing high levels of mIgA+ B cells. In three separate experiments (a, b, c), resting DBA/2 splenic B cells were stimulated with either LPS (20 μg/ml; 2a-left, 2b-left; 2c) or CD40L (10 μg/ml; 2a-right, 2b-right; 2c) in the presence of IL-4 (10,000 U/ml), IL-5 (150 U/ml), αδ-dex (as indicted per figure), and TGF-β (as indicated per figure). Cells were harvested 4 days after initiation of culture for quantitation of mIgA+ B cells by flow cytometric analysis. 15,000 cells from each group were analyzed in 2a and 10,000 cells from each group were analyzed in 2b and 2c.

Determination of Optimal Concentrations of IL-4 and IL-5 for Inducing High Levels of mIgA+ B Cells.

	% mIgA+ cells	
	No TGF-β	+TGF-β
LPS + αδ-dex + IL-4 (10,000 U/ml) + IL-5 (1500 U/ml)	N.D.	8.16
+ IL-5 (150 U/ml)	0.35	10.09
+ IL-5 (15 U/ml)	0.21	N.D.
+ IL-5 (1.5 U/ml)	0.23	3.18
LPS + αδ-dex + IL-4 (1,000 U/ml) + IL-5 (1500 U/ml)	0.61	6.62
+ IL-5 (150 U/ml)	0.31	6.29
+ IL-5 (15 U/ml)	0.40	3.74
+ IL-5 (1.5 U/ml)	0.50	1.26
LPS + αδ-dex + IL-4 (100 U/ml) + IL-5 (1500 U/ml)	0.29	1.70
+ IL-5 (150 U/ml)	0.24	0.95
+ IL-5 (15 U/ml)	0.66	0.44
+ IL-5 (1.5 U/ml)	0.46	0.52
LPS + αδ-dex + IL-4 (10 U/ml) + IL-5 (1500 U/ml)	0.54	0.96
+ IL-5 (150 U/ml)	0.63	0.66
+ IL-5 (15 U/ml)	0.72	0.74
+ IL-5 (1.5 U/ml)	0.90	0.55

Table 1. Resting DBA/2 splenic B cells were stimulated with LPS (20 μg/ml), αδ-dex (3 ng/ml), and various concentrations of IL-4 and IL-5 in the presence or absence of TGF-β (10 ng/ml). Cells were harvested 4 days after initiation of culture for quantitation of mIgA+ B cells by flow cytometric analysis. 10,000 cells from each group were analyzed.

Costimulation with LPS + αδ-dex in the Presence of IL-4, IL-5 and TGF-β Selectively Induces High Levels of mIgA+ B Cells.

	% mIg+ cells	
	TGFβ	
LPS + IL4 + IL5 + αδdex	-	+
mIgA+	0.35	11.25
mIgG1+	9.50	8.99
mIgG2b+	0.33	0.34
mIgG3+	0.27	0.19

TABLE 2. Resting DBA/2 splenic B cells were costimulated with LPS (20 μg/ml), αδ-dex (3 ng/ml), IL-4 (10,000 U/ml), and IL-5 (150 U/ml) in the presence or absence of TGF-β (10 ng/ml). Cells were harvested 4 days after initiation of culture for quantitation of the percentages of B cells bearing mIg of the isotypes indicated. 15,000 cells from each group were analyzed. Data is representative of multiple experiments.

cells generated in response to LPS or CD40L, in the presence or absence of TGF-β, with or without αδ-dex, IL-4, and/or IL-5 (Figure 3). TGF-β stimulated a modest increase in mIgA$^+$ cells in cultures activated with most combinations of stimuli, but these percentages did not exceed 2%. This was consistent with the data reported by Lebman et al (25) and Ehrhardt et al. (27), but inconsistent with the finding of substantial induction of mIgA$^+$ B cells by Sonoda et al in response to LPS + TGF-β alone (289). Only in the combined presence of αδ-dex, IL-4, IL-5, *and* TGF-β were relatively large percentages of mIgA$^+$ cells generated upon activation with either LPS or CD40L (11.0% and 12.3%, respectively).

TGF-β induction of mIgA+ cells is associated with selective stimulation of IgA secretion. To determine if costimulation of resting B cells would result in enhanced levels of IgA secretion, concomitant with the increased percentages of mIgA$^+$ cells, culture supernatants were analyzed for Ig secretion by ELISA. Costimulation of B cells with either LPS + αδ-dex or CD40L + αδ-dex, in the presence of IL-4, IL-5, and TGF-β, stimulated a >34-fold, and a >176-fold induction in IgA secretion respectively, compared to that obtained in the absence of TGF-β (Table 3). This enhancement in IgA secretion was selective in that the concentrations of most other Ig isotypes were significantly suppressed. The modest induction of IgG3 and IgG2b secretion in this system was not substantially affected by the presence of TGF-β. These studies indicate that the factors present in this system, in addition to being sufficient to

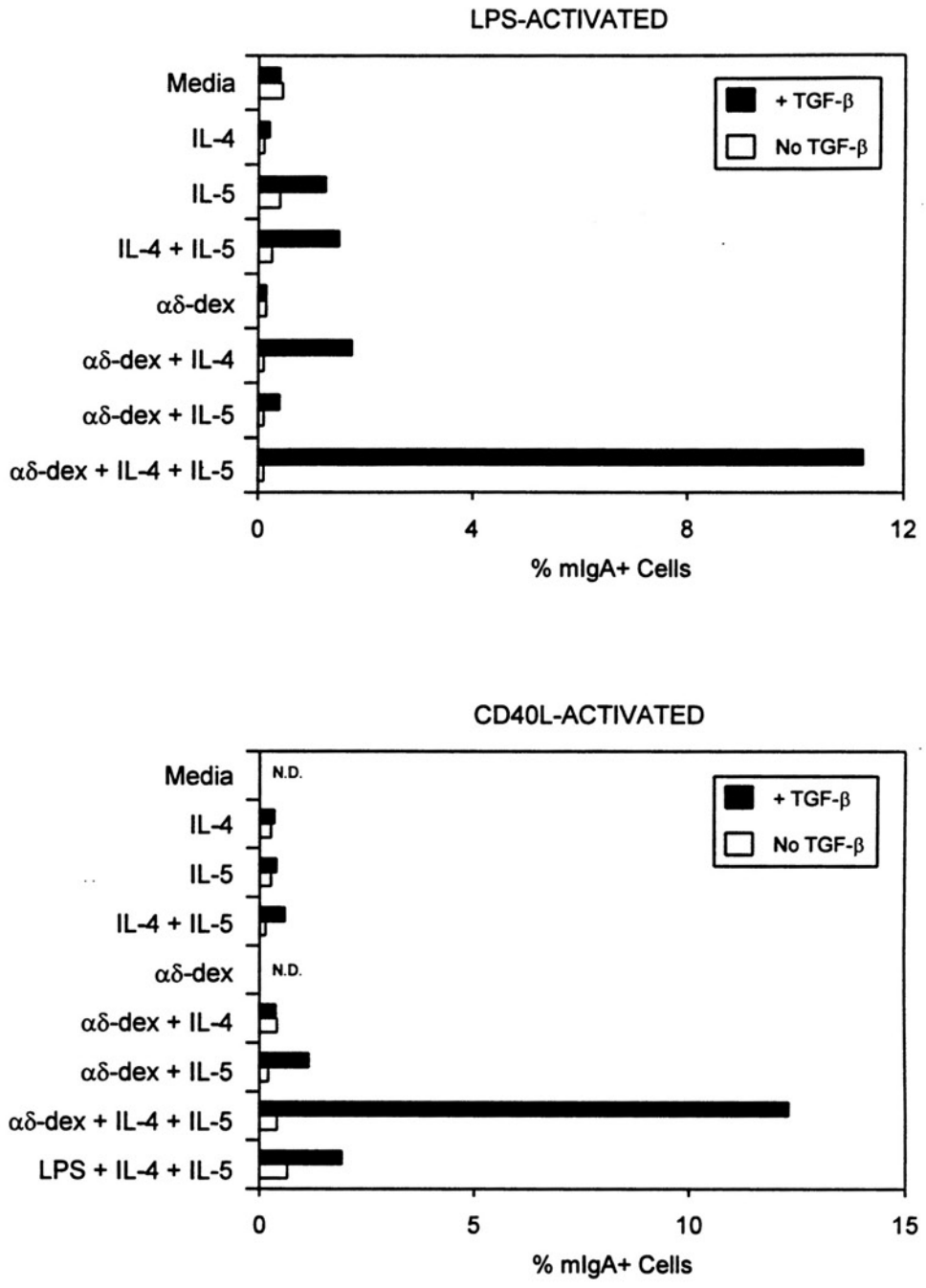


FIGURE 3. IL-4, IL-5 and αδ-dex are all required for optimal generation of mIgA+ cells in response to LPS + TGF-β or CD40L + TGF-β. LPS-activated or CD40L-activated DBA/2 splenic B cells were cultured with various combinations of αδ-dex, IL-4, and/or IL-5, in the presence of absence of TGF-β at concentrations indicated in Figure 1. Flow cytometry was performed to quantitate the percentages of mIgA+ cells 4 days after inititation of culture. Data is representative of three similar experiments.

TGF-β Selectively Stimulates IgA Secretion by LPS- or CD40L-activated Splenic B Cells in the Presence of αδ-dex, IL-4, and IL-5

	Ig isotype secretion (ng/ml)			
	LPS + αδ-dex + IL-4 + IL-5		CD40L + αδ-dex + IL-4 + IL-5	
	No TGF-β	+TGF-β	No TGF-β	+TGF-β
IgM	33,500	3,250	62,000	17,300
IgG3	87	<25	170	85
IgG1	3,160	590	14,00	2,120
IgG2b	25	28	<25	124
IgG2a	<50	<50	<50	<50
IgE	264	<6	288	13
IgA	<25	852	<25	4,400

TABLE 3. Cells were stimulated for 6 days in the presence of the indicated reagents at dosages indicated in Figure 1. Culture supernatants were then harvested for determination of Ig isotype concentrations by ELISA. Each value is the mean of triplicate cultures. Data is representative of multiple experiments.

obtain high levels of mIgA$^+$ cells, also provide sufficient stimulation for obtaining concomitantly high levels of IgA secretion.

αδ-dex strongly augments proliferation of LPS- or CD40L-activated cells and allows for vigorous cell growth in the presence of high concentrations of TGF-β. Since DNA synthesis has been implicated as a key parameter for induction of Ig class switching (160), it is possible that, although TGF-β may *target* the $C_H\alpha$ gene for recombination, as evidenced by its induction of $C_H\alpha$ germline RNA (282), its anti-proliferative effects (195) may decrease the subsequent level of $C_H\alpha$ gene rearrangement and thus prevent high-rate IgA class switching. We hypothesized that if B cells were exposed to a powerful activation stimulus this would result in vigorous proliferation that could overcome the anti-proliferative effects of TGF-β and allow the class switching effects of TGF-β to occur. To determine the contribution made by the individual components in this system on the level of DNA synthesis, we measured [^{3}H]-thymidine ([^{3}H]-TdR) incorporation in response to various combinations of stimuli. In the absence of TGF-β, αδ-dex strongly enhanced [^{3}H]-TdR incorporation by LPS-activated cells (Figure 4a). The combination of IL-4 + IL-5 had little effect on proliferation in response to either LPS or LPS + αδ-dex. In the presence of high concentrations of TGF-β, although DNA synthesis was variably inhibited under all conditions compared to that seen in the absence of TGF-β, only following stimulation with both LPS + αδ-dex, in the presence or absence of IL-4 + IL-5, were relatively high levels of DNA synthesis maintained. These results also show that

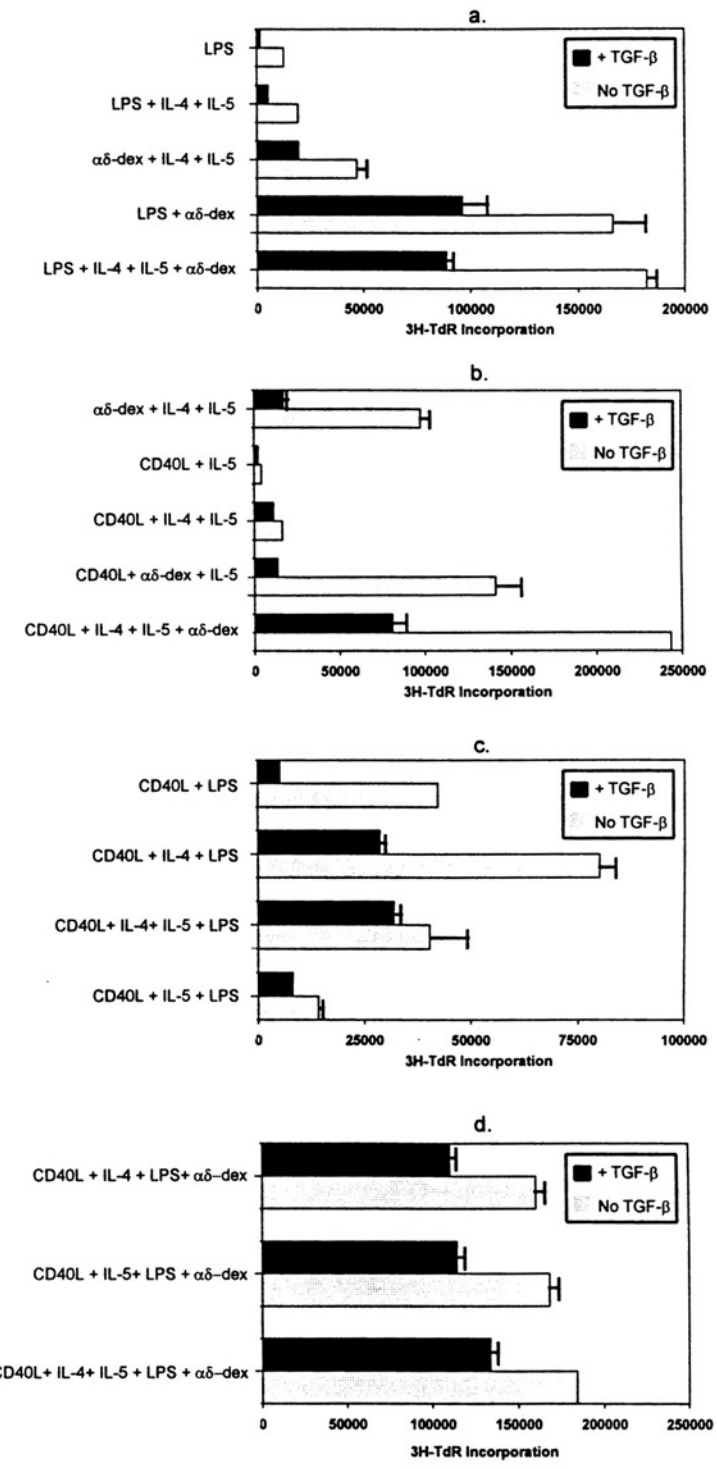


FIGURE 4. αδ-dex strongly augments proliferation of LPS-, CD40L- or CD40L/LPS-activated B cells. Resting DBA/2 splenic B cells were stimulated as indicated. The dosages of each component are the same as that shown in Figure 1. 48h after initiation of culture, wells were pulsed for 4h with ^{3}H-TdR then harvested and analyzed for ^{3}H-TdR incorporation. Data are expressed as the arithmetic mean ± SEM of triplicate cultures. Data are representative of two similar experiments.

costimulation with LPS/αδ-dex plus TGF-β, in the absence of IL-4 and IL-5, is sufficient to enhance proliferation while, in contrast, IL-4 and IL-5 are necessary to obtain high levels of mIgA$^+$ cells.

Similar results were obtained when CD40L was used instead of LPS, however, in this analysis only in the presence of IL-4, IL-5, and TGF-β were relatively high levels of DNA synthesis maintained by CD40L + αδ-dex costimulated cells (Figure 4b). Additionally, for both costimulation systems, LPS/αδ-dex or CD40L/αδ-dex, viable cell yields corresponded to the levels of [^{3}H]-TdR incorporation (Table 4a, 4b).

Costimulation with CD40L and LPS also allowed for relatively high levels of growth in the presence of TGF-β, independent of the combined action of IL-4 and IL-5 (Figure 4c), but did not result in increased percentages of mIgA$^+$ cells (see Figure 1). Therefore, enhanced proliferation is only a partial explanation for the role of αδ-dex in this system. Simultaneous stimulation by all three B cell activators, αδ-dex, LPS, and CD40L, in the presence of TGF-β, and in the independent or combined presence of IL-4 and IL-5, resulted in levels of DNA synthesis nearly equal to that observed in the absence of TGF-β (Figure 4d). These results demonstrate that costimulation of B cells in this manner allows for enhanced viability and cell proliferation in the presence of TGF-β and suggest that this stimulation enables the cells to survive long enough for TGF-β to effect IgA class switching.

The absolute requirement for αδ-dex for high-rate induction of IgA class switching in this system strongly suggested that the precursors of mIgA$^+$ cells in these cultures were mIgD$^+$ at initiation of culture and hence had not already switched to IgA. Further, since flow cytometric studies showed that costimulation with either CD40L or LPS with αδ-dex in the presence of IL-4, IL-5, and TGF-β resulted in >100-fold increases in mIgA$^+$ cells relative to cultures not receiving TGF-β, and since the overall expansion of cells by day 4 of culture was at best only 12-fold (Table 4a, 4b), it is unlikely that the percentages of mIgA$^+$ cells generated in this system could be accounted for solely on the basis of a selective expansion of mIgA$^+$ cells.

Three B cell activators are required for IgA class switching by mIgM+mIgA- B cells. To determine whether costimulation with either LPS/αδ-dex or CD40L/αδ-dex in the presence of IL-4, IL-5 and TGF-β acted directly on splenic B cells to stimulate IgA class switching, highly purified mIgM$^+$mIgA$^-$ cells were isolated by electronic cell sorting and the percentage of mIgA$^+$ cells was compared to that obtained for nonsorted cells following culture with each costimulation system (Figure 5a). As observed previously, costimulation of nonsorted cells with either LPS/αδ-dex or CD40L/αδ-dex in the presence of IL-4 + IL-5 + TGF-β resulted in high percentages of mIgA$^+$ cells (8.0% and 12.3%, respectively). In contrast, much lower percentages of mIgA$^+$ cells were observed following similar costimulation of sorted mIgM$^+$mIgA$^-$ cells. No significant enhancement was observed for either nonsorted or sorted

αδ–dex Strongly Augments Cell Viablility in LPS- or CD40L-activated B Cells in the Presence of High Concentrations of TGF-β

a.

	Viable cells (x 10^3/ml)	
	TGFβ	
	-	+
LPS	211	78
LPS + IL-4 + IL-5	620	51
αδ–dex + IL-4 + IL-5	1373	62
LPS + αδ–dex	540	362
LPS + IL-4 + IL-5 + αδ-dex	1580	1442

b.

	Viable cells (x 10^3/ml)	
	TGFβ	
	-	+
CD40L + IL-4 + IL-5	320	56
CD40L + IL-5 + αδ–dex	1140	22
CD40L + IL-4 + IL-5 + αδ–dex	1920	330
IL-4 + IL-5 + αδ–dex	1400	22

TABLE 4. Resting DBA/2 splenic B cells were stimulated as indicated. The initial cell concentration per well was 1.25 x10^5 cells/ml. On day 4, the cells in the culture wells were resuspended by pipetting, a 100 μl aliquot was removed and mixed with an equal volume of trypan blue. A 10 μl aliquot was placed in a hemocytometer counting chamber and the viable cells were identified on the basis of dye-exclusion. Data is representative of two similar experiments.

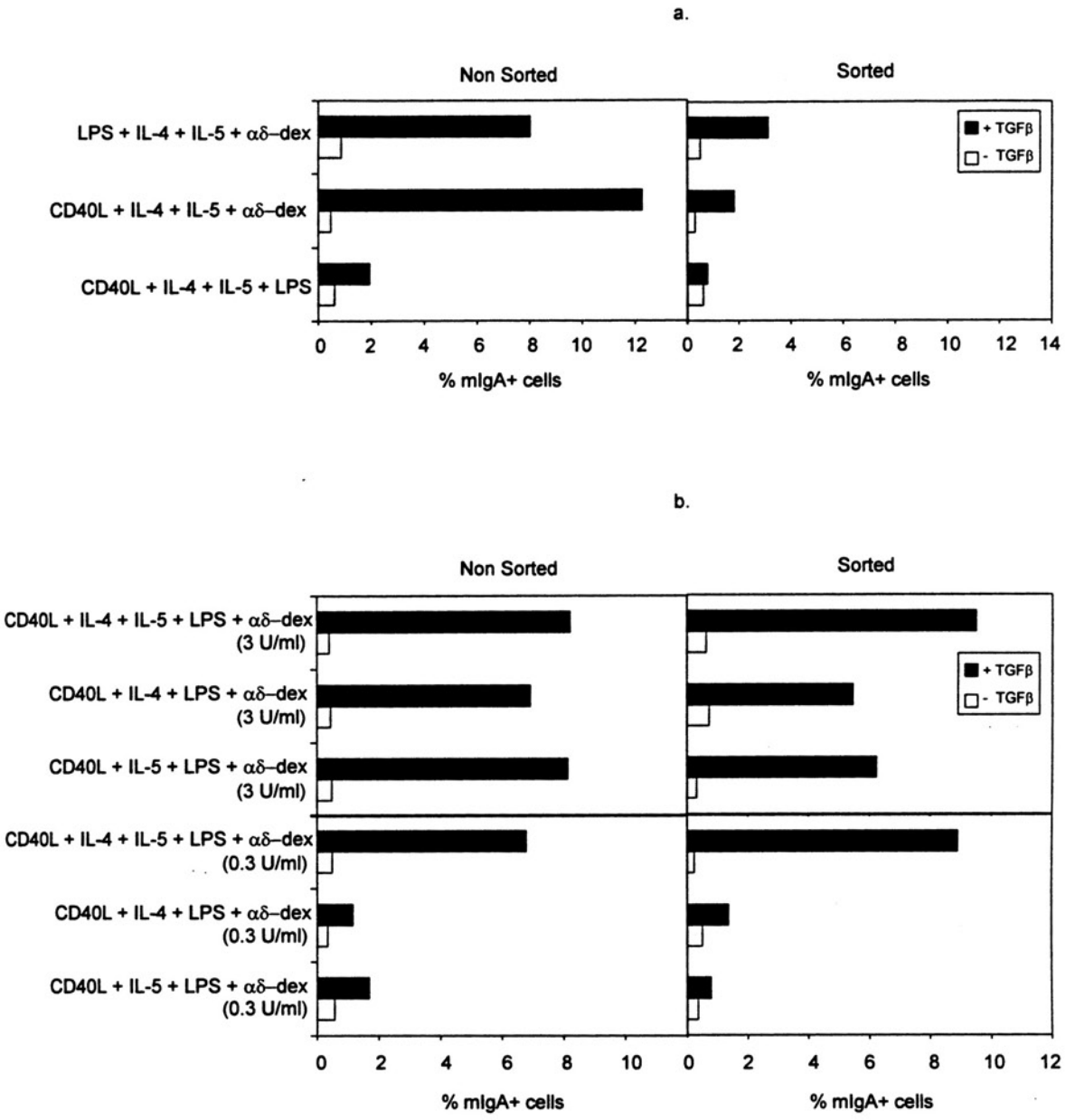


FIGURE 5. Three B cell activators are required for high-rate induction of mIgA by mIgM+ mIgA- B cells. A preparation of resting DBA/2 splenic B cells was divided into two groups. One group of cells was stained with FITC-anti-IgA plus PE-anti-IgM and mIgM+ mIgA- cells were isolated by electronic cell sorting to >99% purity. The second group of cells was not subjected to cell sorting. Both groups were placed in culture simultaneously (1.25×10^5 cells/ml) in the presence of the indicated reagents at dosages described in Figure 1. Cells were harvested 4 days after initiation of culture for quantitation of mIgA+ B cells by flow cytometric analysis. 15,000 cells from each group were analyzed. Data in a and b were generated in the same experiment.

cells following costimulation with CD40L/LPS + IL-4 + IL-5 + TGF-β. Several interpretations of these results exist: 1) both LPS/αδ-dex and CD40L/αδ-dex costimulatory systems may be acting in a post-switch capacity to selectively expand mIgA$^+$ cells but this would imply the unlikely possibility that these mIgA$^+$ cells co-expressed mIgD, since αδ-dex was necessary in this system, 2) sorting could have eliminated a critical cell type necessary for optimal IgA class switching, 3) these results could merely be an artifact of the sorting process, although this was thought to be a less likely possibility, since sorted cells in other studies from our lab behaved similarly to non-sorted cells in response to certain stimuli which act directly at the B cell level.

Further analysis of additional combinations of B cell activators in this experiment revealed that sorted cells could accomplish high-rate IgA class switching in the presence of three B cell activators (LPS, αδ-dex, and CD40L). In contrast, stimulation with all three activators did not further enhance switching by nonsorted cells over that observed in the presence of two B cell activators (Figure 5b). Furthermore, in the presence of LPS + CD40L, and higher concentrations of αδ-dex (3 ng/ml), a significant induction in mIgA$^+$ cells was observed even in the absence of IL-4 or IL-5, by nonsorted and sorted cells, when compared with that obtained with lower αδ-dex concentrations (0.3 ng/ml) (Figure 5b). These results further demonstrate the complexity of *in vitro* IgA class switching and suggest that 1) nonsorted cell populations may include a population of non-B, non-T cells that deliver a key activation signal necessary

for IgA class switching that can be substituted for by the additional, third, B cell activator and 2) that costimulation with all three B cell activators may provide additional signals, relative to that observed with two activators, that can substitute for IL-4 and IL-5 in this system. However, the presence of all three activators while restoring high-rate IgA class switching in the sorted B cell population, did not further enhance IgA switching in the non-sorted group.

IL-2 can substitute for IL-5 in stimulating IgA class switching by B cells activated by LPS/αδ-dex but not by CD40L/αδ-dex. The multiparameter requirements for induction of IgA class switching may not be exclusive. Thus, it was possible that other cytokines could substitute for one or more of the key components in this system. Since IL-5 is both a B cell maturation factor for Ig secretion as well as a key contributor to the class switch process (290), I tested whether another well-known B cell maturation factor, IL-2, could substitute for IL-5 in inducing IgA class switching. Stimulation by either LPS + αδ-dex or CD40L + αδ-dex in the presence of IL-4, IL-5, and TGF-β resulted in relatively high percentages of $mIgA^+$ cells as was typically observed (Figure 6). Addition of IL-2 to either of these costimulatory systems did not significantly augment IgA class switching. However, when splenic B cells were costimulated with LPS/αδ-dex in the presence of IL-4, TGF-β, and IL-2 instead of IL-5, percentages of $mIgA^+$ cells were similar to that observed following LPS + αδ-dex + IL-4 + IL-5 + TGF-β stimulation. In contrast, no enhancement in percentages of $mIgA^+$ cells were observed following stimulation with CD40L + αδ-dex + IL-4

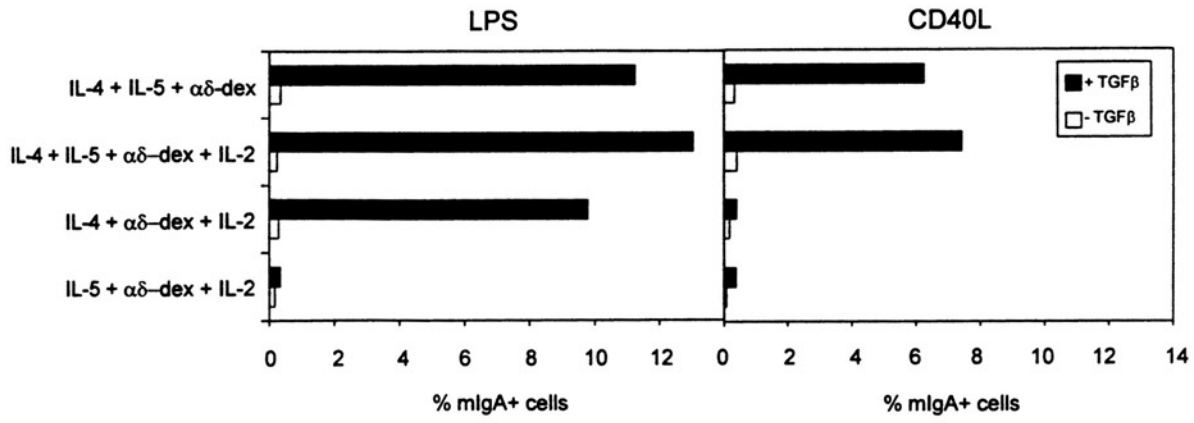


FIGURE 6. IL-2 can substitute for IL-5 in stimulating IgA class switching by B cells activated by LPS/αδ–dex but not CD40L/αδ-dex. Resting DBA/2 splenic B cells were costimulated with LPS/αδ–dex or CD40L/αδ-dex in the presence or absence of the IL-4 and/or IL-5, TGF-β, and IL-2 (150 U/ml). LPS, CD40L, αδ–dex, IL-4, IL-5, and TGF-β were used at dosages described in Figure 1. Cells were harvested 4 days after initiation of culture for quantitation of mIgA+ B cells by flow cytometric analysis. 15,000 cells from each group were analyzed. Data is representative of two similar experiments.

+ IL-2 + TGF-β (Figure 6). Substitution of IL-2 for IL-4 did not enhance IgA class switching by B cells activated by either costimulatory system (Figure 6) indicating that IL-4 and IL-5 acted in distinct, though cooperative, ways to mediate IgA class switching. While the basis for these results will require further study, one potential explanation for the preferential action of IL-2 in the LPS costimulatory system could be that LPS/αδ-dex costimulation selectively upregulates, or CD40L/αδ-dex costimulation selectively inhibits, the expression of IL-2 receptors on the surface of the activated B cell while the expression of IL-5 receptors are regulated similarly by both modes of costimulation. This could, therefore, result in enhanced responsiveness of LPS/αδ-dex-stimulated B cells, but not CD40L/αδ-dex-stimulated cells, to the differentiation activities of IL-2. Alternatively, IL-2 may simply fail to integrate its signals with those mediated through CD40 in order to achieve the IgA switch.

IL-10 suppresses LPS/αδ-dex- but not CD40L/αδ-dex-stimulated IgA class switching in the presence of IL-4, IL-5, and TGF-β. IL-10 has previously been implicated in playing an important role in stimulating IgA class switching in humans (292). To investigate the potential role of IL-10 in murine IgA class switching, B cells were costimulated with either LPS/αδ-dex or CD40L/αδ-dex ± IL-4 ± IL-5 ± IL-10 and TGF-β. The addition of IL-10 to cells costimulated with CD40L/αδ-dex in the presence of IL-4, IL-5, and TGF-β resulted in a modest enhancement in the percentage of $mIgA^+$ cells from that observed in the absence of IL-10 (Figure 7).

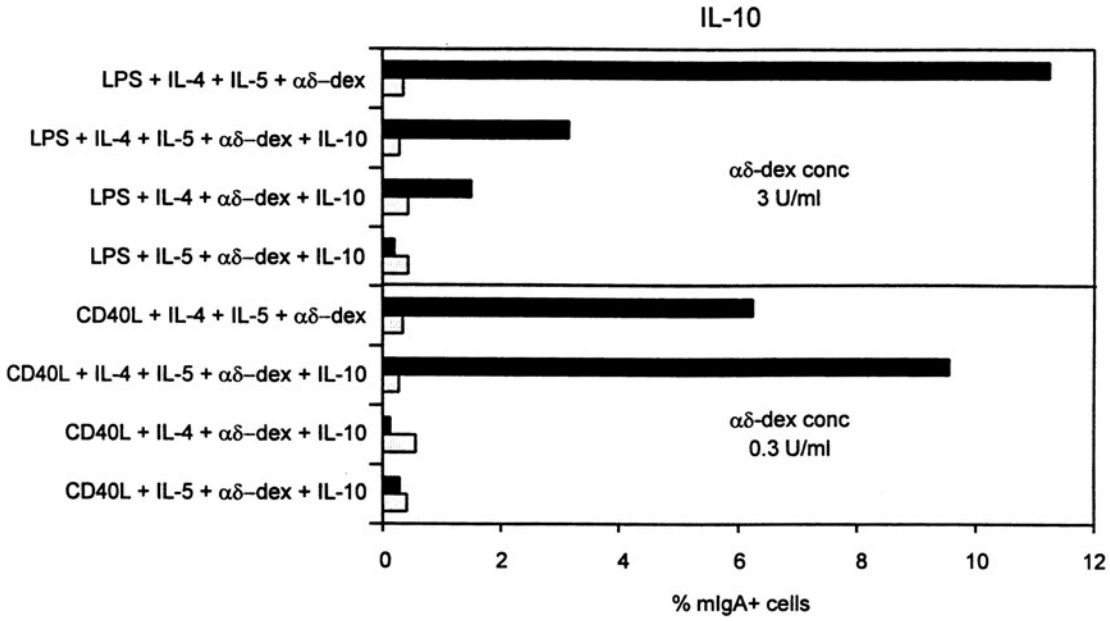


FIGURE 7. IL-10 suppresses LPS/αδ–dex- but not CD40L/αδ-dex-stimulated IgA class switching in the presence of IL-4, IL-5, and TGF-β. Resting DBA/2 splenic B cells were stimulated with LPS/αδ–dex or CD40L/αδ-dex in the presence or absence of IL-4 and/or IL-5, TGF-β, and IL-10 (30 ng/ml). LPS., CD40L, αδ–dex, IL-4, IL-5, and TGF-β were used at dosages described in Figure 1. Cells were harvested 4 days after initiation of culture for quantitation of mIgA+ B cells by flow cytometric analysis. 15,000 cells from each group were analyzed. Data is representative of two similar experiments. (■ + TGF-β, ▨ - TGF-β)

In contrast, the percentage of mIgA$^+$ cells were suppressed 3.5-fold following the addition of IL-10 to cells costimulated with LPS/αδ-dex + IL-4 + IL-5 + TGF-β. In either costimulatory system, IL-10 was not able to substitute for IL-4 or IL-5 for the induction of high-rate IgA class switching. The mechanism underlying the differential effects of IL-10 on LPS- vs CD40L-driven IgA class switching will require further study. Neverthless, the inhibition of murine IgA class switching by IL-10 was in surprising contrast to the stimulatory actions of IL-10 on IgA secretion reported for human B cells.

IFN-γ suppresses IgA class switching. Since IFN-γ inhibits most IL-4-mediated effects on murine B cells (reviewed in 294), and since IL-4 was a key component for inducing IgA class switching we tested whether IFN-γ could suppress the generation of mIgA$^+$ cells in this system (Figure 8). Thus, resting splenic B cells were stimulated for high-rate IgA production by culture in either LPS or CD40L + αδ-dex + IL-4 + IL-5 + TGF-β. To these cultures varying concentrations of IFN-γ, ranging from 0.3-100 U/ml, were added. IFN-γ strongly inhibited the percentage of mIgA$^+$ cells generated in response to activation with either LPS or CD40L in the presence of αδ-dex, IL-4, IL-5 and TGF-β. Optimal inhibition of IgA class switching occured at 100 U/ml of IFN-γ representing an ~4-fold and ~6-fold suppression for CD40L- and LPS-activated cells, respectively. A significant reduction in mIgA$^+$ cells was observed with as little as 1-3 U/ml of IFN-γ.

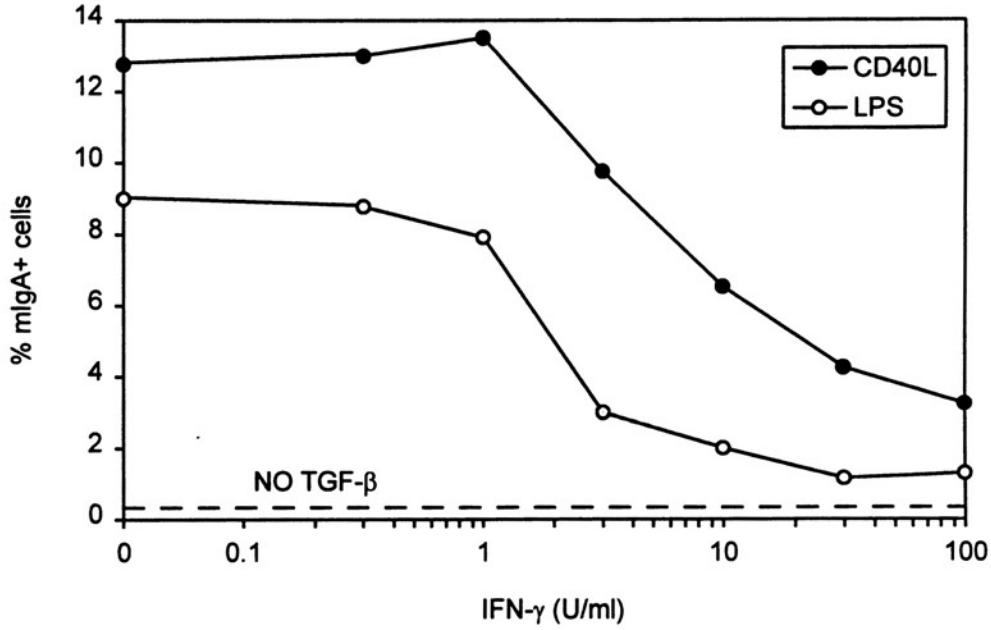


FIGURE 8. IFN-γ suppresses the generation of mIgA+ cells. LPS- or CD40L- activated DBA/2 splenic B cells + αδ-dex, IL-4, IL-5, TGF-β (at dosages described in Figure 1) were cultured with varying concentrations of IFN-γ (0.3-100 U/ml). The percentages of mIgA+ cells were quantitated 4 days after initiation of culture by flow cytometry. 15,000 cells were analyzed per group. (—) represents the average percentage of mIgA+ cells present in control groups containing no TGF-β.

SUMMARY

The mechanisms underlying the requirement for the combined actions of αδ-dex, IL-4, and IL-5 with LPS + TGF-β or CD40L + TGF-β for inducing relatively high-rate IgA class switching are unknown. Recent studies have revealed several regions within the Ig locus which may regulate Ig class switching and could in theory be modulated by one or more of the stimuli used in this system. Thus, investigation on the effect of these stimuli on germline $C_H\alpha$ transcription (295), germline $C_H\alpha$ RNA stability (296), the intronic IgH enhancer (297), the 3' α enhancer (157), and switch region binding proteins (298) are warranted.

IV. INDUCTION OF IgG2b CLASS SWITCHING BY TRANSFORMING GROWTH FACTOR-β

INTRODUCTION

IgG2b is known to be induced during the course of an immune response to some bacterial and viral infections (299). The ability of IgG2b to fix complement, and to bind Fc receptors for IgG found on B cells, macrophages, NK cells, and neutrophils suggests that it could play an important role in opsonization and phagocytosis of invading microorganisms (300). Studies have also shown that IgG2b selectively inhibits LPS-induced Ig secretion by binding to FcγRII on macrophages which leads to the release of prostaglandin E_2 (PGE_2), an arachidonic acid metabolite with immunoinhibitory properties (301,302). Thus, IgG2b may also function to down-modulate established immune responses to specific antigenic stimuli.

Bacterial LPS had been shown previously to induce IgG3 class switching and to a much lesser extent, the switch to IgG2b (1,7). No cytokine, however, had been described which could regulate IgG2b class switching in a positive fashion. Therefore, the initial experiments in this section were designed to identify a cytokine which might function to selectively induce IgG2b switch recombination in LPS-activated B cells. In this regard, a previous report had indicated that TGF-β was a switch factor for the IgA class (23). Of interest, in this study whereas TGF-β caused a substantial inhibition in the concentrations of most LPS-induced Ig isotypes

secreted into the culture supernatant it failed to reduce the concentrations of IgG2b (23). On this basis initial experiments focused on the possibility that TGF-β, while functioning as an IgA switch factor might additionally regulate the expression of IgG2b in a positive fashion. The earlier observations that IL-4 could coinduce IgG1 (1,2,3,14,15,88) and IgE (13,14,17,303,304) class switching and that IFN-γ (17-22) could stimulate class switching to both IgG3 (136) and IgG2a established a precedent that TGF-β might also co-regulate two Ig isotypes.

RESULTS AND DISCUSSION

A. TGF-β is a switch factor for the IgG2b subclass

The relative and absolute levels of IgG2b secreted in response to LPS activation *in vitro* differs depending upon the strain of mouse from which the B cells were derived. Initial studies demonstrated that LPS-activated resting B cells from BALB/c mice secreted up to 10-fold less IgG2b, relative to IgG1 and IgG3, when compared to B cells obtained from the DBA/2 strain (Table 5). IgM secretory responses by BALB/c-derived B cells were roughly comparable or only somewhat lower than those from DBA/2 mice. Since BALB/c-derived B cells secreted relatively low amounts of IgG2b in response to LPS we chose this strain of mouse to test the hypothesis that TGF-β was a switch factor for the IgG2b subclass.

LPS-activated BALB/c-derived B Cells Synthesize Relatively Low Levels of IgG2b.

	Ig secretion			
Strain	IgG2b	IgG3	IgG1	IgM
		ng/ml		
BALB/c	85	5,000	310	52,500
DBA/2	850	1,750	24	125,000

TABLE 5. B cells from each strain were cultured at 10^5/ml in the presence of LPS for 6 days. Culture supernatants were then removed for measurement of Ig isotype concentrations by ELISA. This data is representative of four similar experiments.

TGF-β selectively stimulates the secretion of IgG2b as well as IgA by LPS-activated B cells. Resting B cells were stimulated with LPS in the absence or presence of log increments of TGF-β (0.001-1 ng/ml) and the concentrations of secreted IgM and IgG2b in the culture supernatants (SN) were measured 6 days later by ELISA (Figure 9a). This time point for measuring the concentrations of secreted Ig isotypes was chosen because previous work from our laboratory indicated that no further Ig secretion was observed beyond 6 days of culture with LPS. TGF-β induced a dose-dependent increase in the concentration of secreted IgG2b in LPS-activated B cell cultures (Figure 9a). The highest level of induction of IgG2b secretion occurred at 1 ng/ml of TGF-β which represented a 19-fold enhancement over that seen in the absence of TGF-β stimulation. Stimulation of IgG2b secretion was still observed at 0.1 ng/ml TGF-β whereas 0.01 ng/ml was without effect. In separate experiments it was observed that 3 ng/ml of TGF-β was relatively ineffective at stimulating IgG2b secretion by LPS-activated cells (data not shown) most likely reflecting its strong anti-proliferative effect. By contrast the addition of TGF-β led to a dose dependent inhibition of IgM secretion in LPS-activated cultures with the highest degree of suppression occuring at 1 ng/ml of TGF-β and representing an ~2-fold reduction.

IL-5 is known to stimulate B cell maturation to Ig secretion (305-307). Although this effect of IL-5 is relatively Ig isotype-non-specific, some selectivity for induction of IgA secretion has been reported (26, 287-289, 308). Indeed a previous report indicated

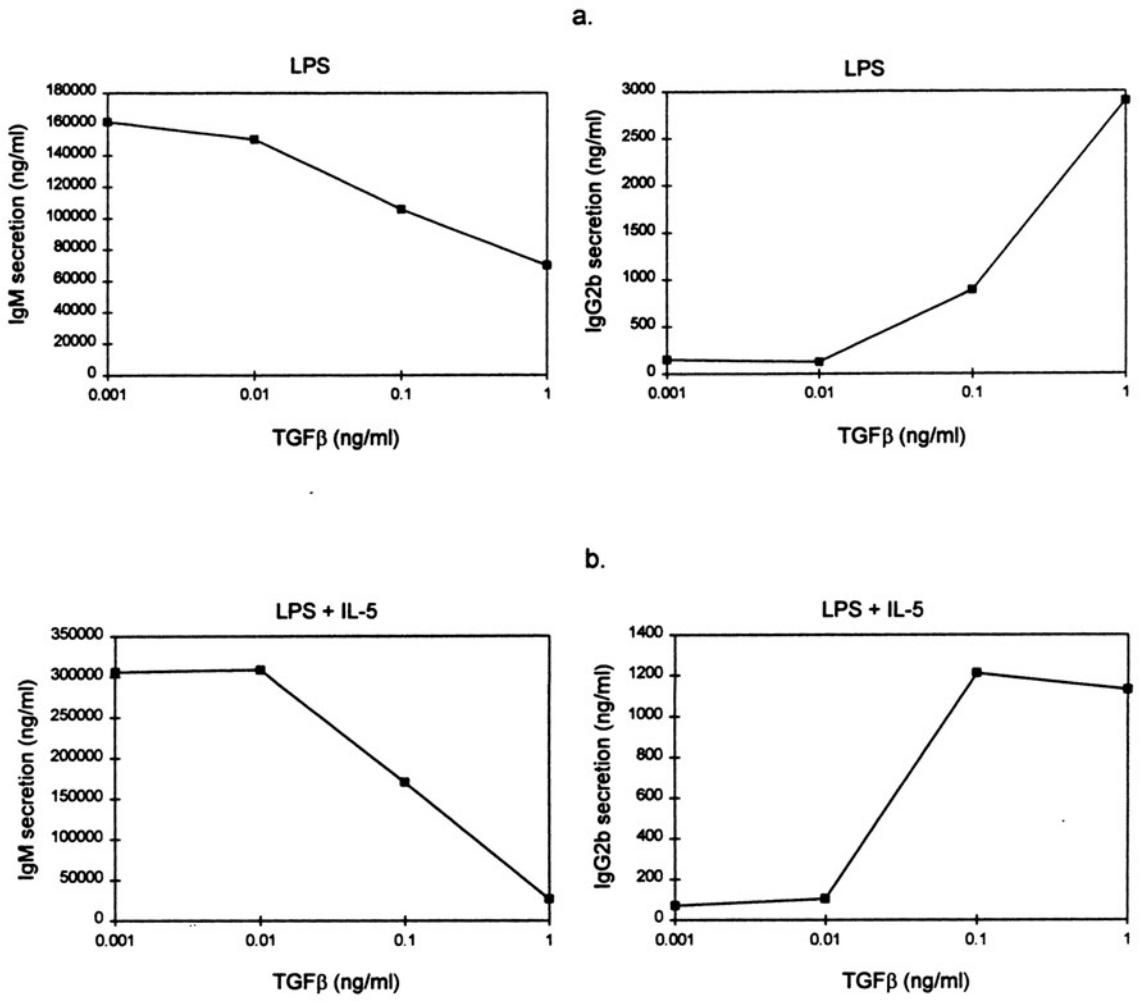


FIGURE 9. TGF-β stimulates the secretion of IgG2b by LPS- or LPS + IL-5-activated B cells. Resting BALB/c (final concentration 1.25 x 10^5 cells/ml) were stimulated with LPS (20μg/ml) and log increments of TGF-β (0.001-1 ng/ml) in the absence (a) or presence (b) of IL-5 (150 U/ml). On day 6 after initiation of culture, the concentrations of secreted IgM and IgG2b in the culture supernatants were measured by ELISA

that the combination of IL-5 and TGF-β led to selectively higher levels of IgA secretion in LPS-activated cultures, relative to that observed with TGF-β alone (26). Thus log increments of TGF-β (0.001-1 ng/ml) were added to LPS + IL-5-activated resting B cell cultures to determine whether higher levels of enhancement of IgG2b secretion in response to TGF-β could be obtained (Figure 9b). TGF-β stimulated up to a 17-fold enhancement in IgG2b secretion by LPS+IL-5-activated cells relative to cultures stimulated with LPS+IL-5 alone. IgG2b induction was once again observed at 0.1 and 1.0 ng/ml of TGF-β with no effect seen at 0.01 ng/ml TGF-β. Likewise TGF-β inhibited IgM secretion by LPS-activated B cells in the presence of IL-5. Since IL-5 did not appreciably affect the TGF-β-mediated stimulation of IgG2b secretion by LPS-activated cells, it was not studied further in LPS-activated cultures.

To better assess the selectivity of the TGF-β-mediated enhancement of the IgG2b secretory response, the concentrations of other Ig isotypes (IgG3, IgG1, IgG2a, and IgA) in addition to IgM and IgG2b were measured in LPS-activated cultures, in the presence or absence of 1 ng/ml of TGF-β. As observed above, TGF-β enhanced IgG2b secretion and suppressed the synthesis of IgM (Figure 10). As reported previously, TGF-β also stimulated the secretion of IgA, although in our hands this induction was always quite modest in the absence of additional exogenous stimuli. By contrast, TGF-β either inhibited or left unaffected the concentrations of secreted IgG3 IgG1, and IgG2a. Furthermore, TGF-β did not induce detectable IgE secretion (data not shown).

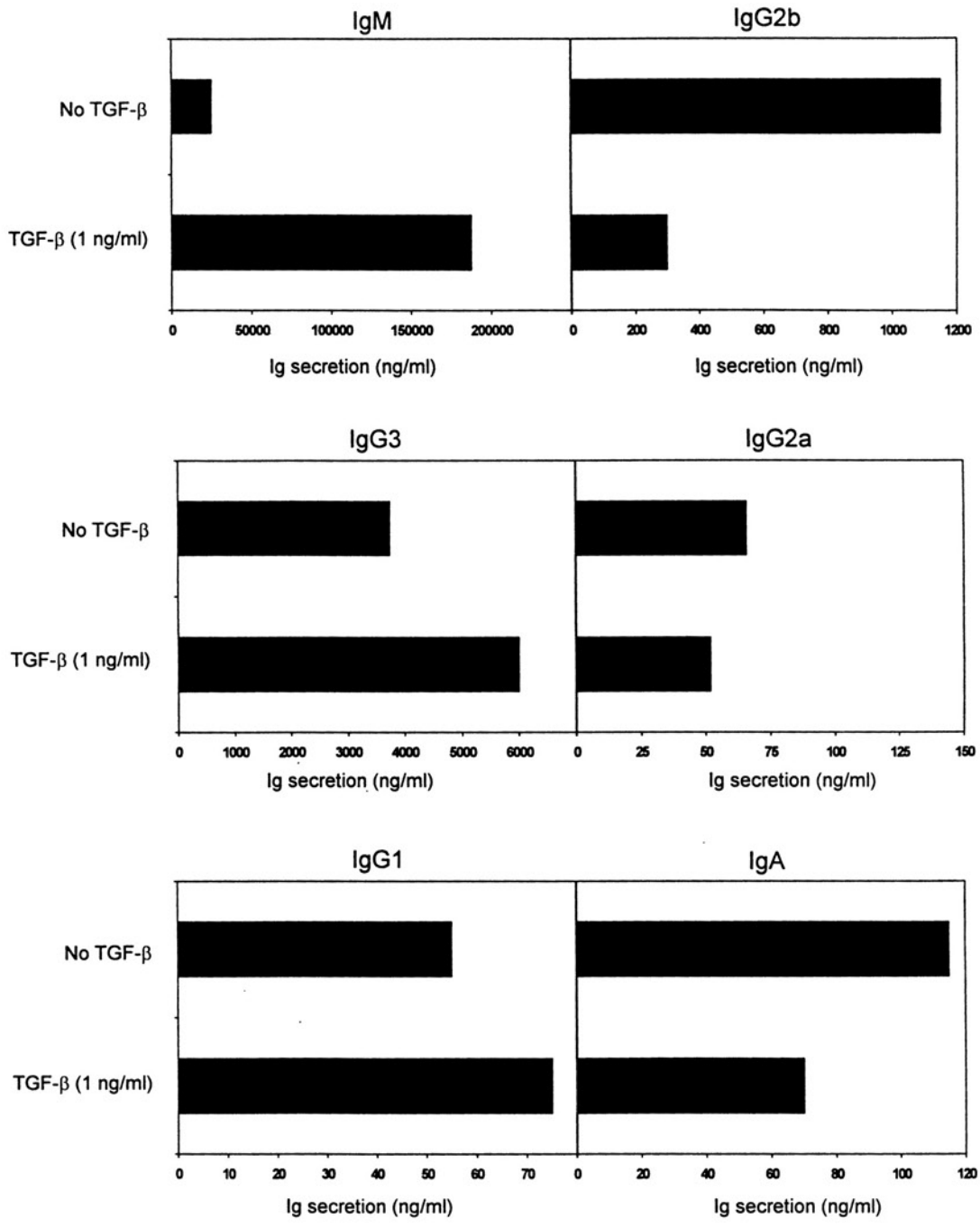


FIGURE 10. TGF-β selectively stimulates the secretion of IgG2b as well as IgA, by LPS-activated B cells. Resting BALB/c B cells (final concentration, 3.75×10^5 cells/ml) were stimulated with LPS (20μg/ml) in the presence or absence of various concentrations of TGF-β (0.03-3 ng/ml). On day 6 after initiation of culture, the concentrations of secreted Ig isotypes (as indicated) were measured by ELISA.

IgG2b induction is a specific effect of TGF-β. To establish the specificity of the TGF-β-mediated enhancement in IgG2b secretion we tested the ability of a neutralizing anti-TGF-β mAb to abrogate the TGF-β-mediated induction of IgG2b (Table 6). The addition of TGF-β led to an ~10-fold enhancement in IgG2b secretion in LPS-activated cultures and this was inhibited by anti-TGF-β mAb, but not an isotype matched control mAb. The modest suppression of IgM secretion by TGF-β was reversed by anti-TGF-β mAb further indicating that the anti-TGF-β mAb itself was not non-specifically suppressive in LPS-activated cultures. Although TGF-β appeared to have a slight enhancing effect on IgG3 secretion this was not reversed with anti-TGF-β mAb.

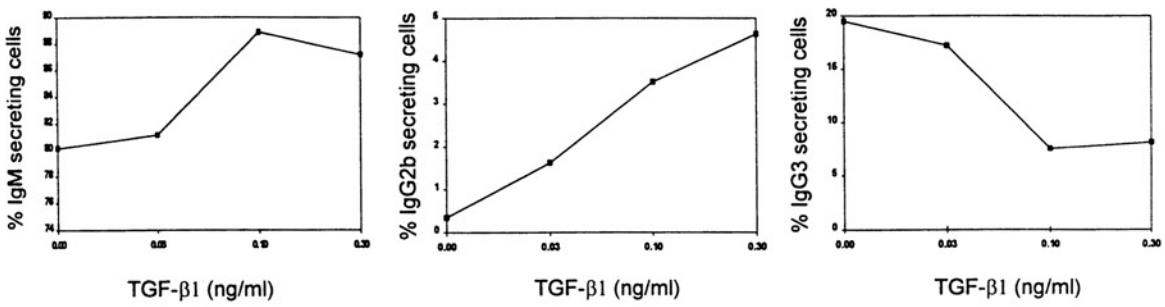
TGF-β stimulates an increase in the percentage of IgG2b-secreting cells in LPS-activated cultures. In the next series of studies we wished to determine whether the TGF-β-mediated stimulation of IgG2b secretion was due to an increase in the number of IgG2b-secreting cells and/or the amount of IgG2b secreted per cell. The number of Ig isotype-secreting cells generated in response to TGF-β in LPS-activated cultures was determined in three separate experiments, in collaboration with Dr. Dennis Klinman, FDA (Bethesda, MD) using an ELISPOT assay (Figures 11a/11b, and 12a). ELISPOT analysis was performed on cells 4.5 days after initiation of culture, a time that was previously shown to be optimal for detecting Ig-inducing cells. When expressed as the percentage of total Ig-secreting cells at a given concentration of TGF-β, a striking dose-dependent increase was observed for IgG2b-secreting cells with

Anti-TGF-β mAb Specifically and Selectively Inhibits IgG2b Secretion by B Cells Activated with LPS Plus TGF-β1

	Ig secretion		
Strain	IgG2b	IgG3	IgM
		ng/ml	
LPS	130	1,250	47,900
LPS plus TGF-β1	1,260	2,810	28,900
LPS plus TGF-β1 plus anti- TGF-β	265	2,140	53,900
LPS plus TGF-β plus control mAb	1,650	3,500	29,400

TABLE 6. BALB/c-derived B cells were stimulated for 24 h at 5 x 10^5/ml in the presence of LPS (40 μg/ml). 24 h later TGF-β1 (final concentration 1.0 ng/ml) with or without anti-TGF-β (10 μg/ml final concentration) or control mAb, MB86 (10 μg/ml final concentration) were added to culture in an equal volume. Culture supernatants were removed 6 d after initiation of culture for measurement of Ig isotype concentrations by ELISA.

a.



b.

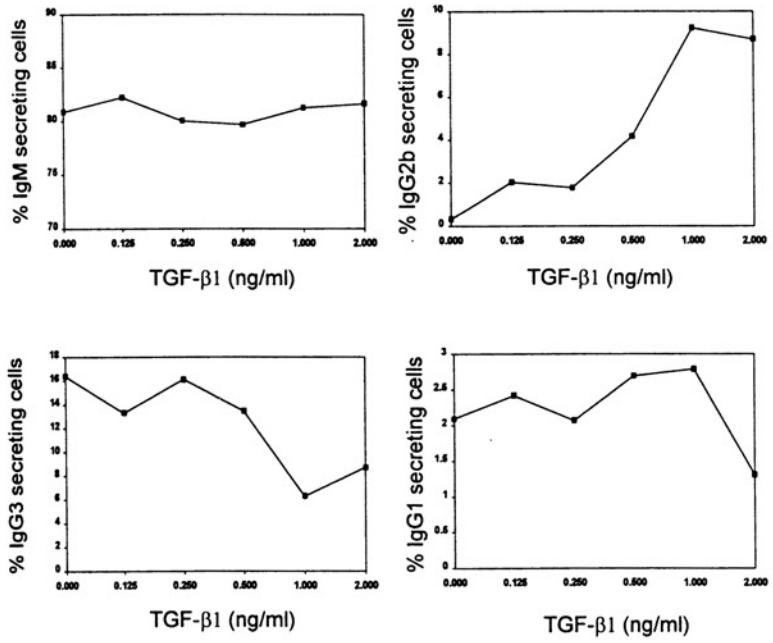


FIGURE 11. TGF-β stimulates an increase in the percentage of IgG2b-secreting cells in LPS-activated cultures. BALB/c splenic B cells were cultured for 24 h with LPS (40μg/ml) at 5 x 10^5 cells/ml in a volume of 5 ml of medium to which varying concentrations of TGF-β1, also in 5 ml of medium, were added to achieve the indicated final concentration. Cells were harvested 4.5 d after initiation of culture for quantitation of Ig isotype-secreting cells by ELISPOT assay.

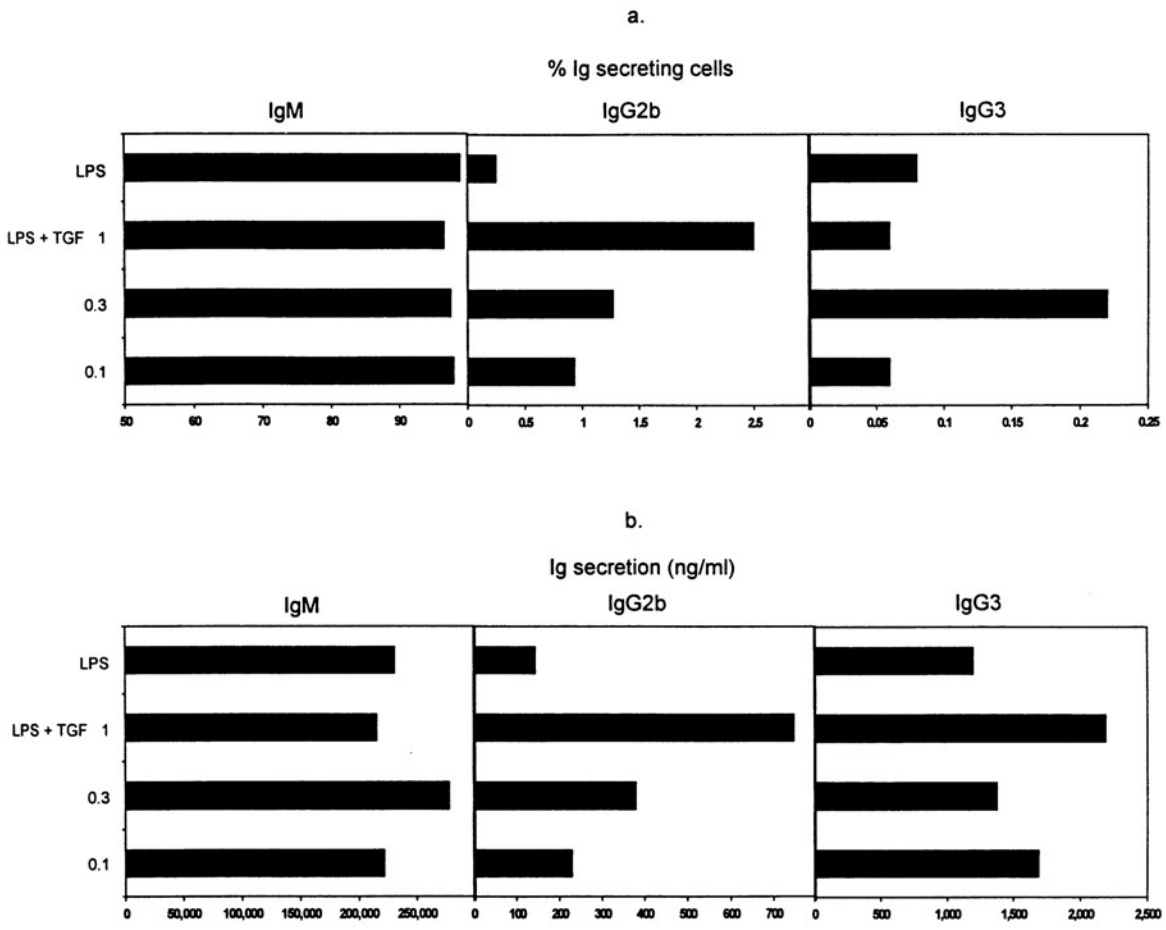


FIGURE 12. TGF-β stimulates a concomitant increase in the concentration of secreted IgG2b and the percentage of IgG2b-secreting cells in LPS-activated cultures. BALB/c splenic B cells were cultured for 24 h with LPS (40 μg/ml) at 5 x 10^5 cells/ml in a volume of 5 ml of medium to which varying concentrations of TGF-β1, also in 5 ml of medium, were added to achieve the indicated final concentration. Duplicate culture plates were prepared for separate analysis for percentage of IgG2b-secreting cells and for IgG2b secretion. For (a), cells were harvested 4.5 d after initiation of culture for quantitation by ELISPOT assay. For (b) culture supernatants were removed 6 d after initiation of culture for measurement of Ig isotype concentration by EILISA.

a maximal increase of 30-fold observed with 1.0 ng/ml of TGF-β (Figure 11a/b, Figure 12a). Enhancement in percentages of IgG2b-secreting cells for all three experiments ranged from 10-30-fold, with 1.0 ng/ml of TGF-β producing optimal levels in each experiment. In contrast, the percentage of cells secreting IgM, IgG3, or IgG1 either remained relatively unchanged or decreased in the presence of increasing concentrations of TGF-β relative to that seen with LPS alone. 1.0 ng/ml of TGF-β induced a 5.4-fold and an 18-fold increase in the absolute number of IgG2b-secreting cells relative to that observed with LPS alone (Figure 11a/11b). In contrast, the absolute numbers of cells secreting IgM, IgG3, and IgG1 decreased at 1.0 ng/ml of TGF-β by 5.5-, 14.8-, and 4.2-fold, respectively. The concentration of secreted IgG2b, as measured by ELISA, increased >5-fold in the presence of 1.0 ng/ml of TGF-β (Figure 12a) compared to that obtained following stimulation with LPS alone, and was concomitant with the increase in the percentage of IgG2b-secreting cells at this same concentration of TGF-β. This increase in IgG2b secretion and percentage of IgG2b-secreting cells was accompanied by an unchanged level of IgM secretion (Figure 12b) and percentage of IgM secreting cells (Figure 12a). The average amount of IgG2b produced per IgG2b-secreting cell, following stimulation by TGF-β at a concentration which optimally increased IgG2b secretion and the percentage of IgG2b-secreting cells, was 3.5-fold less than that produced in the absence of TGF-β (Table 7). This indicated that TGF-β had an inhibitory effect on B cell maturation to Ig secretion. Further addition of TGF-β to LPS-activated cultures typically led to a reduction in viable cell yields, consistent with its known anti-

The Amount of IgG2b Produced per IgG2b-secreting cell is Decreased for LPS-activated B Cells in the Presence of TGF-β

		pg/ml / # secreting cells		
		IgM	IgG2b	IgG3
	LPS	1469	358	8889
LPS + TGFβ	1	764	103	13506
	0.3	1247	130	2680
	0.1	966	105	12504

TABLE 7. The level of Ig produced per Ig-secreting cell was calculated by dividing the concentration (pg/ml) of Ig (from figure 12b) by the total number of Ig isotype specific-secreting B cells observed in the same experiment (data not shown).

proliferative effect. Thus, the net enhancement in total IgG2b secretion in response to TGF-β reflects a balance between the positive TGF-β-mediated selection of the IgG2b subclass and its negative effects on cell outgrowth and maturation to Ig secretion.

TGF-β fails to stimulate IgG2b secretion by B cells activated with dextran-conjugated anti-IgD antibodies (αδ-dex). Resting B cells, activated through the membrane (m)Ig signalling pathway with αδ-dex, proliferate and in the presence of IL-5, secrete relatively large amounts of IgM. Work from our lab has indicated that the mode of B cell activation can play a determining role in the B cell response to cytokines (7). We thus wished to test whether TGF-β could enhance IgG2b secretion by αδ-dex-activated cells, as it does for LPS-stimulated cultures. As assessed by both ELISA (Figure 13a/b) and ELISPOT assays (Figure 13c), titered amounts of TGF-β failed to induce detectable IgG2b secretion or an increase in IgG2b-secreting cells in αδ-dex-stimulated cultures in the presence of IL-5. This further supported the notion of a critical role for the B cell activator in determining the qualitative, as well as quantitative outcome of cytokine-driven response.

TGF-β acts on mIgM+mIgG2b- cells to stimulate IgG2b secretion in LPS-activated cultures. The ability of a cytokine to stimulate the secretion of a particular Ig isotype could reflect its abiliy to induce the switch to that Ig class. Alternatively, although lacking precedent, the cytokine could seletively expand cells already expressing that particular Ig isotype. One way to distinguish

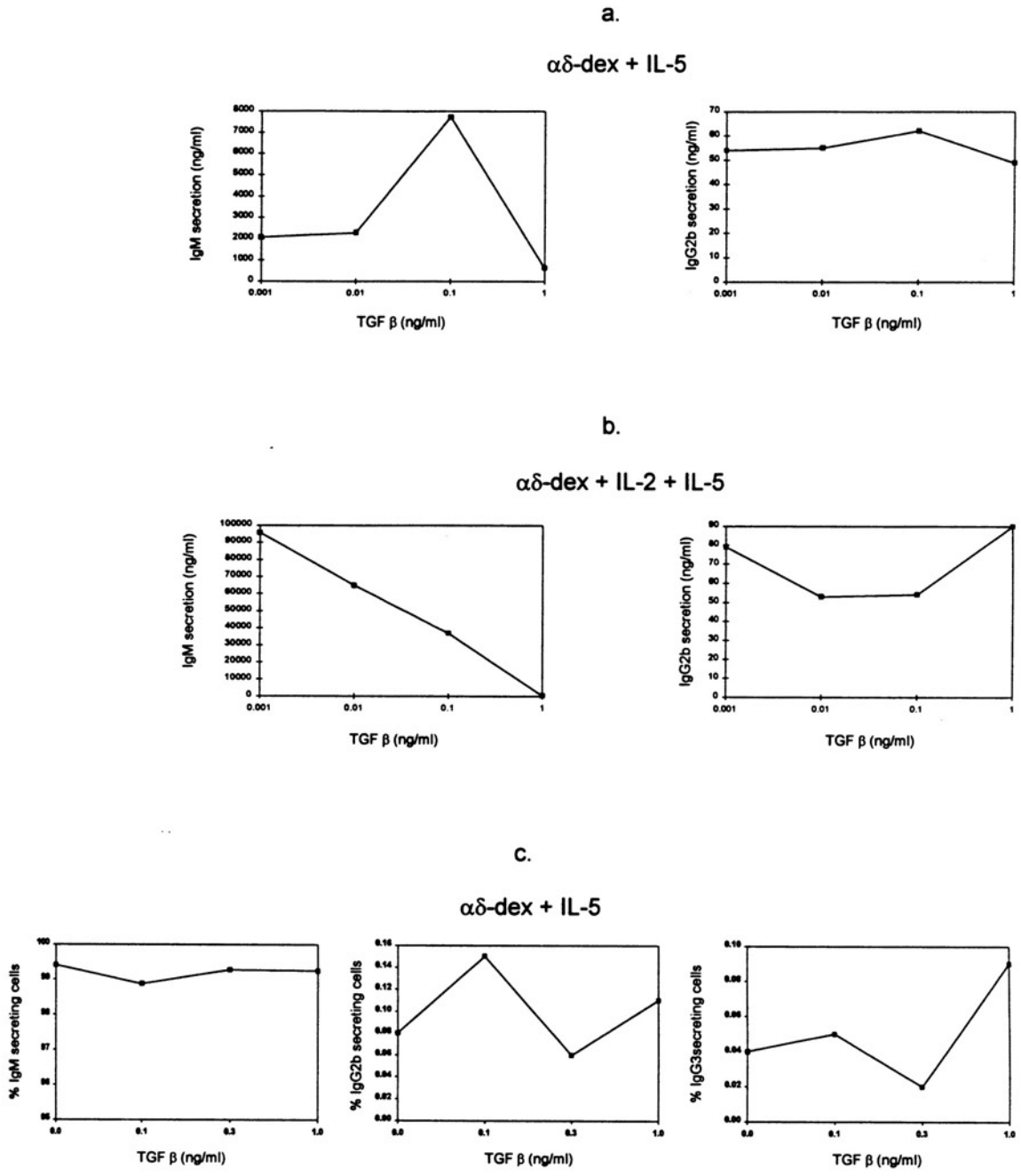


FIGURE 13. TGF-β fails to stimulate IgG2b secretion or the percentage of IgG2b-secreting cells by αδ-dex-activated B cells. In two separate experiments (expt. 1-a,b; expt. 2-c), resting BALB/c B cells were cultured with αδ-dex (3 ng/ml) in the presence of IL-2 (b; 150 U/ml) and/or IL-5 (a, b, c: 150 U/ml) in the presence or absence of various concentrations of TGF-β as indicated. For analysis of the concentration of secreted IgM and IgG2b (a, b), cells were cultured at 1.25 x 10^5 cells/ml. Culture supernatants were harvested on day 6 after initiation of culture and analyzed by ELISA. For analysis of the percentage of Ig-secreting cells (c), 5 x 10^5 cells/ml in a volume of 5 ml of medium were cultured for 24 h prior to the addition of various concentrations of TGF-β1, also in 5 ml of medium. The final concentrations of TGF-β are as indicated. 4.5 d after initiation of culture, cells were harvested for quantitation by ELISPOT assay.

between these possibilities is to obtain highly purified (>99%) $mIgM^+mIgG2b^-$ cells by electronic cell sorting and stimulate with LPS in the presence or absence of TGF-β. Thus resting B cells were stained with a combination of FITC-anti-IgG2b + PE-anti-IgM antibodies and sort-purified $mIgM^+mIgG2b^-$ cells were obtained. TGF-β stimulated a 9-fold enhancement in IgG2b secretion in sorted LPS-activated B cells relative to cells stimulated with LPS alone (Table 8). Additionally, this level of induction was similar to that seen for non-sorted B cells (6-fold) further suggesting that TGF-β acts directly on the B cell and not indirectly through another cell type to enhance IgG2b secretion.

TGF-β selectively enhances the steady-state levels of germline γ2b RNA in LPS-activated cultures. To further assess whether TGF-β was acting as a switch factor for IgG2b, RNA was assayed by Northern blot analysis or S1 nuclease protection assay to determine if TGF-β selectively induced germline C_H γ2b RNA in LPS-activated B cells (Figure 14). This analysis is considered to be the most critical for demonstrating that a cytokine induces an Ig class switch since an increase in the steady state levels of germline C_H RNA specific for a particular C_H gene typically precedes class switching to the expression of that gene by making that gene accessible to the switch recombination machinery (134-137). TGF-β induced a 2.5-fold increase in the steady-state levels of germline C_Hγ2b RNA in LPS-activated B cells while concomitantly reducing by 2.5-fold the levels of germline C_Hγ3 (Figure 14). This further

TGF-β Acts Directly on mIgM$^+$mIgG2b$^-$ B Cells to Stimulate IgG2b Secretion

	Nonsorted			mIgM$^+$ mIgG2b$^-$		
	IgM	IgG2b	IgG3	IgM	IgG2b	IgG3
			ng/ml			
LPS	119,000	86	1,800	175,000	290	2,150
LPS + TGF-β	46,250	500	3,000	106,000	2,600	4,250

TABLE 8. Small splenic B cells were prepared and divided into two groups. One group was stained with FITC-anti-IgG2b plus PE-anti-IgM, mIgM$^+$mIgG2b$^-$ cells were isolated by electronic cell sorting to >99% purity. The second group was not subjected to cell sorting. Cells from both groups were placed in culture simultaneously (final concentration 5 X10^5 cells/ml) in the presence of LPS (40 μg/ml). 24 h later, an equal volume of medium with or without 1.0 ng/ml of TGF-b was added. Culture supernatant was removed 6 d after initiation of culture and Ig isotype concentrations were measured by ELISA.

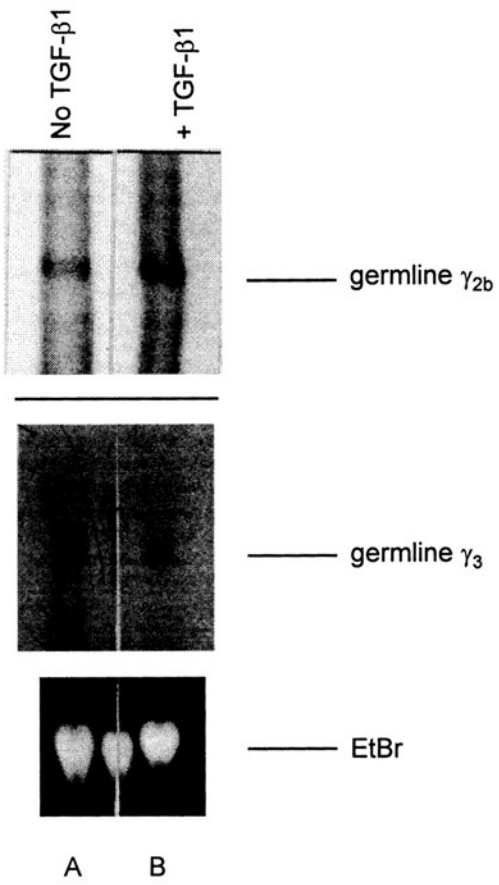


FIGURE 14. TGF-β1 selectively stimulates an increase in the steady-state levels of germline CHγ2b mRNA by LPS-activated B cells. Resting splenic BALB/c-derived B cells were cultured for 24 h in the presence of LPS (40 μg/ml) and either anti-TGF-β1 mAb (1D11.16.8; 10 μg/ml), to neutralize any endogenous TGF-β1, or an isotype-matched control mAb (MB86; 10 μg/ml) at 5 x 10^5 cells/ml in 25 ml of medium. An equal volume of TGF-β1 (2.0 ng/ml) or medium alone was added to each culture on day 1. Cells were harvested 3 d after initiation of culture and mRNA was extracted for quantitation of steady-state levels of germline CHγ2b by S1 nuclease protection assay or CHγ3 by Northern blot analysis. Ethidium bromide (EtBr) staining of RNA is included to show that nearly equal quantities of RNA were loaded and transferred per group.

supported the notion that TGF-β is a switch factor for the IgG2b subclass in LPS-activated B cells.

SUMMARY

These studies demonstrate that TGF-β: 1) selectively stimulates IgG2b, as well as IgA, secretion by LPS-activated cells and that this activity is specific in that it is reversed with a neutralizing anti-TGF-β monoclonal antibody, 2) selectively enhances the percentage as well as the absolute number of IgG2b secreting cells, 3) stimulates IgG2b secretion by cells which are $mIgM^{+}mIgG2b^{-}$ at initiation of culture, 4) selectively upregulates the levels of steady-state germline γ2b transcripts, and 5) fails to act as an IgG2b switch factor in cells activated through the mIg signalling pathway utilizing αδ-dex. Thus this work strongly supports the view that TGF-β promotes IgG2b class switching *in vitro*. Furthermore, these studies emphasize the importance of the mode of B cell activation, in combination with the specific stimulatory cytokine, for inducing the class switch to a particular Ig isotype.

B. Studies to assess a role for endogenous TGF-β in regulating *in vivo* IgG2b class switching

To assess the physiologic relevance of the *in vitro* observation that TGF-β is a switch factor for the IgG2b subclass in LPS-activated B cell cultures, we wished to determine if *in vivo* IgG2b production is

TGF-β-dependent. It was previously demonstrated that LPS stimulates TGF-β production by both B cells and macrophages. Furthermore, immunization of mice with LPS leads to a strong LPS-specific humoral immune response. Thus, studies were carried out to answer the following questions: 1) Does immunization with LPS induce elevated serum levels of LPS-specific IgG2b, and if so, 2) is this enhancement dependent upon endogenous TGF-β?

Immunization of mice with LPS induces serum LPS-specific IgM and IgG2b antibodies. Studies were first aimed at determining if LPS immunization induced serum LPS-specific IgG2b titers and at assessing the concentration of LPS which elicited optimal increases in LPS-specific IgG2b. LPS at either 0.1, 1, or 10 μg was injected i.v. into BALB/c mice. Mice were bled on the 6th and 9th day following LPS injection and the serum was analyzed for LPS-specific IgM and IgG2b by ELISA. Five mice per group were tested and the data is expressed as the reciprocal of the serum titer yielding an optical density (O.D.) equal to a titer point defined as the highest O.D. obtained for unimmunized control mice. Optimal increases in serum LPS-specific IgG2b were obtained by the 6th day following injection of 10 μg of LPS with little significant change in titers on day 9 (Figure 15a). The serum level of LPS-specific IgG2b decreased in a dose-dependent fashion with the highest IgG2b levels obtained with 10 μg of LPS, moderate levels with 1 μg, and undetectable levels following injection of 0.1 μg of LPS. Equivalent serum titers of LPS-specific IgM were observed after immunization with 10 and 1 μg of LPS and undetectable titers were observed at 0.1 μg of LPS.

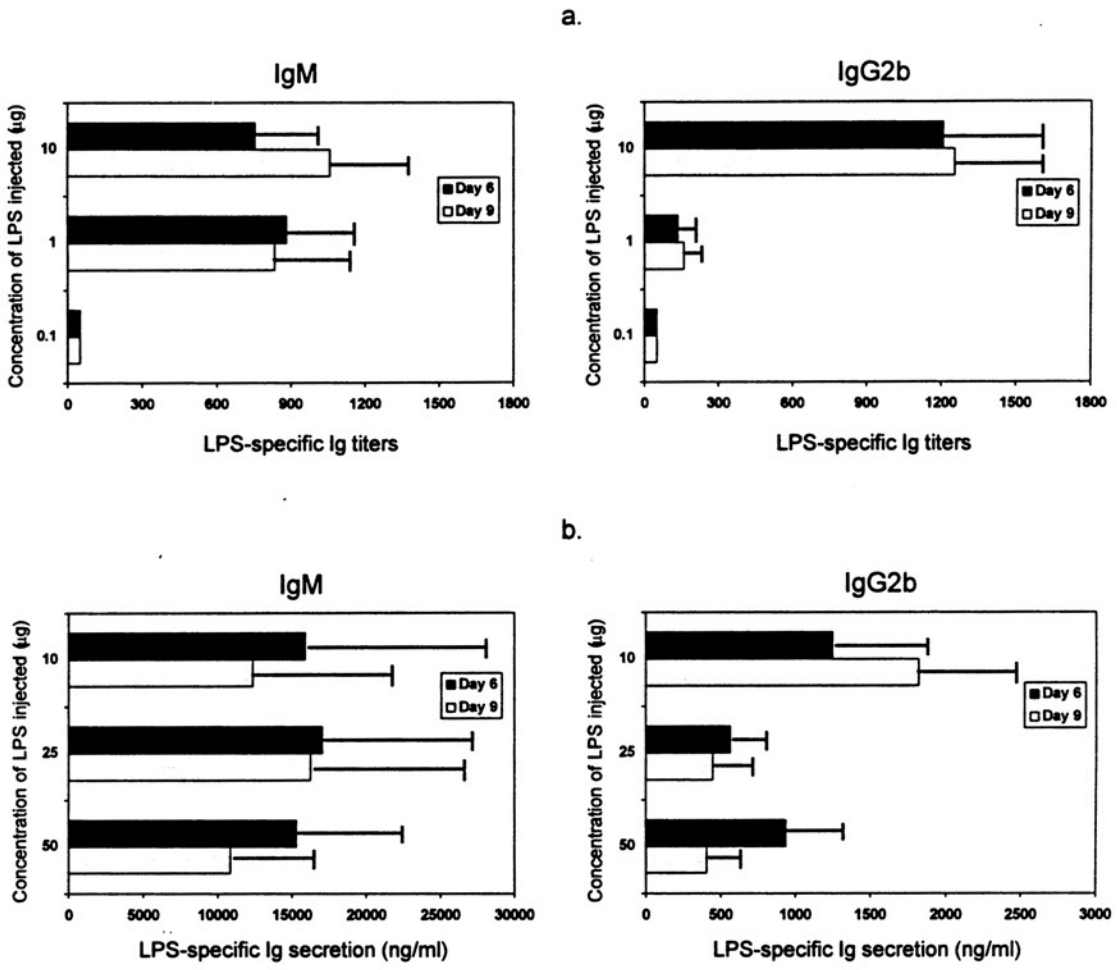


FIGURE 15. LPS-immunization of mice induces serum LPS-specific IgM and IgG2b. BALB/c mice were immunized with various lower (a) and higher (b) concentrations of LPS as indicated. Serum samples were obtained prior to immunization (prebleed) and on the 6th and 9th day after immunization. Analysis of serum LPS-specific Ig titers (a, explained in text) or concentration (b, ng/ml) was accomplished by LPS-specific ELISA. The data is expressed as the mean ± SEM for five mice tested per group and is representative of multiple experiments.

To determine whether 10 μg of LPS elicited optimal IgG2b secretion, mice were injected with higher doses of LPS and assessed for serum LPS-specific IgM and IgG2b titers (Figure 15b). BALB/c mice were injected with either 10, 25, or 50 μg of LPS i.v. Mice were bled on the 6th and 9th day following LPS injection and serum samples were analyzed by LPS-isotype specific ELISA. At the time this titration was performed, we acquired LPS-isotype specific monoclonal antibodies of known concentration which were serially diluted and used to generate a standard curve for each Ig isotype tested. Hence, the data is expressed as ng/ml of LPS-specific Ig isotype present in each serum sample. Again, optimal levels of IgG2b secretion were observed by the 6th day following injection with 10 μg of LPS with little change seen on day 9 (Figure 15b). The two higher concentrations of LPS, 25 μg and 50 μg, generated 1-4.5-fold less serum IgG2b on either day 6 or day 9 than that observed with 10 μg of LPS while IgM secretion was relatively constant for all three LPS concentrations tested. Thus, LPS induction of LPS-specific IgM and IgG2b antibody titers provided a model system for studying a potential role of endogenous TGF-β in the generation of these *in vivo* IgG2b responses.

Co-injection of LPS with neutralizing anti-TGF-β mAb fails to alter the induction of serum LPS-specific IgG2b titers. To evaluate a potential *in vivo* role of endogenous TGF-β in the LPS induction of serum LPS-specific IgG2b antibody, BALB/c mice were immunized with 10 μg of LPS with or without anti-TGF-β mAb injected one day prior and 4 days after LPS immunization. As

before LPS immunization elicited an induction in serum LPS-specific IgM and IgG2b titers (Figure 16). However, co-injection of anti-TGF-β did not alter the serum titers of these Ig isotypes. The inability to block *in vivo* IgG2b secretion with this antibody could be explained by (1) the possible presence of additional TGF-β isoforms, active in IgG2b induction, not neutralized by this antibody, (2) TGF-β-independent pathways for induction of IgG2b class switching in response to LPS, or (3) insufficient *in vivo* neutralization of endogenous TGF-β with even the high doses of this mAb that were used. This latter explanation could, in part, be a result of upregulated TGF-β transcription by various cell types following treatment with anti-TGF-β or due to enhanced conversion from the latent form of TGF-β to the active form in various cell types following anti-TGF-β treatment.

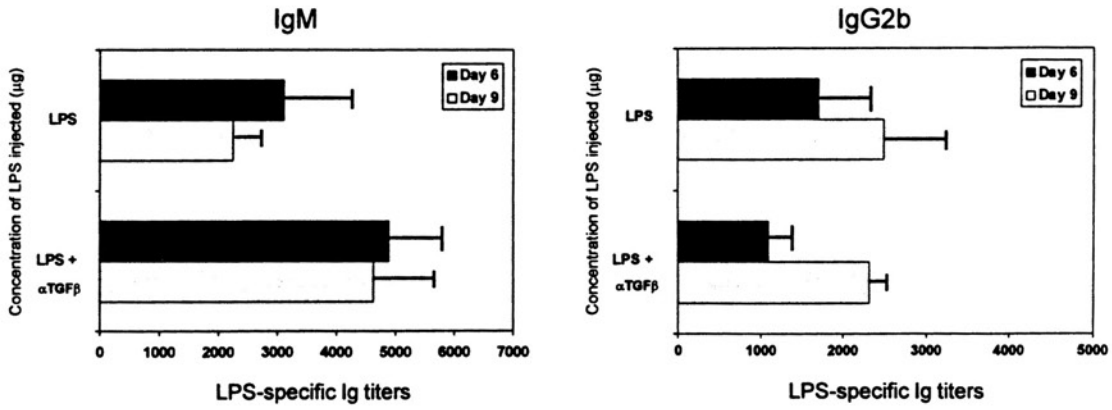


FIGURE 16. Co-injection of LPS with neutralizing anti-TGF-β mAb fails to alter the induction of serum LPS-specific IgG2b titers.. BALB/c mice were immunized with LPS alone or with anti-TGF-β mAb (2mg) 1 day prior and 4 days after LPS (10 µg) immunization. Serum samples were obtained and analyzed as indicated in Figure 15. Data is expressed as the reciprocal of LPS-specific Ig titers (see text) and is representative of multiple experiments.

V. INDUCTION OF IgG3 SECRETION BY INTERFERON-γ: A MODEL FOR T CELL-INDEPENDENT CLASS SWITCHING

INTRODUCTION

The ability of polysaccharide antigens, such as those present in abundance in bacterial cell walls, to stimulate *in vivo* Ig secretory responses in T cell-deficient (nude) mice led to the classification of these antigens as T cell-independent (TI) (39-42). These non-mitogenic antigens were further distinguished from the mitogenic antigens, such as LPS, by their inability to elicit humoral responses in mice carrying an X-linked immunodeficiency gene, xid (85,309-314). This led to the subdivision of TI antigens into TI-1 (e.g. LPS) and TI-2 (e.g. polysaccharides) (309,319,315,316). TI-2 (polysaccharide) antigens are distinguished by the presence of multiple, repeating identical antigenic epitopes (59,317) . Hence they are capable of extensively crosslinking and signalling through mIg on B cells. A unique feature of these antigens is their elicitation of antigen-specific IgG3 secretory responses *in vivo* although the cellular basis for this isotype selection is unknown (64). The ability of IgG3 to self-aggregate upon binding antigen leads to high avidity, cooperative binding of this IgG subclass to polysaccharides and hence potentially more efficient clearance of polysaccharide-containing bacteria (41, 318). Until this point no cytokine had been described which regulated the IgG3 subclass in a positive manner.

The study of B cell responses to TI-2 antigens have been hampered by the low frequency of B cells specific for a given immunizing antigen (107). Thus, little is known regarding the specific parameters that regulate the humoral immune response to TI-2 antigens. Mond and colleagues circumvented this problem by creating an *in vitro* polyclonal model system for mIg-mediated signalling by TI-2 antigens through the synthesis of dextran-conjugated anti-IgD antibodies (αδ-dex) (107). αδ-dex polyclonally activates resting murine mIgD$^+$ B cells *in vitro* in a manner which is similar to the specific antibody response induced by the prototypical TI-2 antigen, TNP-Ficoll. Thus, αδ-dex induces resting B cells to proliferate, and in the presence of a differentiation factor, such as IL-5, costimulates the secretion of large amounts of polyclonal Ig, mostly of the IgM class (7). Using this method of B cell stimulation, we set out to determine whether IgG3 could be selectively elicited in response to a particular cytokine(s).

RESULTS AND DISCUSSION

Effects of Th1 and Th2 supernatants on Ig production by αδ-dex- and LPS-activated B cells. Initial studies in our laboratory compared αδ-dex and LPS for their ability to stimulate *in vitro* cytokine-driven Ig isotype production using cytokine-rich supernatant (SN) from two murine CD4$^+$ T cell clones: 1) A.E7, characterized as a Th1 clone based on its secretion, after activation, of IFN-γ and IL-2, but not IL-4, and 2) D10.G4.1 (D10), established as a Th2 clone based on its secretion of IL-4, but not IFN-γ or IL-2,

following similar stimulation. Thus, resting B cells were stimulated with αδ-dex or LPS in the presence of Th1 or Th2 SN (Table 9). αδ-dex-activated B cells secreted large amounts of IgM in the presence of either Th1 or Th2 SN. Th1 but not Th2 SN stimulated large amounts of IgG2a by αδ-dex-activated B cells based upon their presence of IFN-γ, while in contrast, Th2 SN, which contained IL-4 stimulated 14-fold more IgG1 than Th1 SN. Of particular note, Th1 SN, in the presence of the B cell differentiation factor IL-2, stimulated IgG3 secretion to levels 3-fold higher than that observed for LPS alone, whereas Th2 SN was relatively ineffective. These studies suggested that a factor contained within the Th1 supernatant might independently function to induce IgG3 class switching.

IFN-γ selectively induces IgG3 secretion by B cells activated with αδ-dex + IL-5. Since Th1 but not Th2 SN contains IFN-γ (275, 276), and since IFN-γ had previously been shown to stimulate IgG2a class switching in LPS-activated B cells (260), we determined whether this cytokine might also account for the large IgG3 secretory response of αδ-dex-activated B cells to Th1 SN. Although both IL-5 and IL-2 were shown to induce Ig secretion by αδ-dex-activated B cells, data from our lab had indicated that, whereas IL-5 was acting directly on the B cell to induce Ig secretion, IL-2 was acting through a contaminating non-B, non-T cell type. Furthermore, IL-2 was known to induce *endogenous* IFN-γ, presumably from NK cells (248), in the B cell-enriched spleen cell populations used in these studies. For these reasons we chose to use IL-5 as the differentiation-inducing cytokine in this system. Hence,

Ig Isotype Secretory Profile of αδ-dex and LPS-activated B Cells Stimulated with Activated TH1 and TH2 Supernatants.

	Ig Secretion (ng/ml)								
	IgM			IgG2a			IgG1		
	Med	LPS	αδ-dex	Med	LPS	αδ-dex	Med	LPS	αδ-dex
Med	<100	33,800	<100	<5	42	<5	<6	15	<6
Th1 SN	550	18,800	103,000	86	800	1,100	<6	10	700
Th2 SN	390	6,250	60,000	<5	<5	<5	21	5,250	10,000

	IgG3			IgG2b		
Med	<6	550	<6	<6	220	<6
Th1SN	<6	100	1,800	<6	145	19
Th2 SN	<6	15	25	<6	12	<6

TABLE 9. Purified resting splenic B cells were cultured at 1.25 x 10^5 /ml in the presence of LPS (20 μg/ml), αδ-dex (0.01 μg/ml), or medium (Med). Supernatant from activate Th1 (A.E7) and Th2 (D10) clones were additionally added to selected wells at a final concentration of 20%. IL-2 (150 U/ml) was added to cultures receiving Th1 supernatant. Culture supernatants were harvested 6 days after initiation of culture for measurement of Ig isotype concentrations by ELISA. Similar results were obtained in two additional experiments. Adapted from Snapper et. al. (reference 7) with permission.

these studies were directed at determining whether the additional presence of IFN-γ could skew the resulting Ig isotype pattern towards IgG3, as well as IgG2a.

The addition of IFN-γ to αδ-dex + IL-5-activated B cells resulted in decreased IgM secretion with a concomitant 6-fold increase in IgG3 (Figure 17). To analyze this effect of IFN-γ more carefully log increments of IFN-γ were added at the initiation of culture in combination with αδ-dex + IL-5 (Figures 18a and 18b). In both experiments, IL-5 stimulated a large IgM secretory response by αδ-dex-activated B cells, and stimulated much smaller amounts of IgG3 and IgG1. Upon addition of IFN-γ, a dose-dependent increase in IgG3 secretion was obtained and was optimal when cells were cultured with 10-100 U/ml of IFN-γ, with enhancements of IgG3 secretion between 6- and 17-fold relative to that observed with αδ-dex + IL-5 alone. Significant increases in IgG3 secretion were still observed with concentrations of IFN-γ as low as 1 U/ml, although this enhancement was more variable between experiments. Concentrations of IFN-γ below 0.1 U/ml did not enhance IgG3 secretion. IFN-γ-mediated stimulation of IgG3 secretion by αδ-dex + IL-5-activated B cells was accompanied by a strong induction of IgG2a synthesis and suppression of IgM and IgG1 secretion (Figure 18b).

These initial studies demonstrated that IFN-γ selectively stimulated IgG3, as well as IgG2a secretion by αδ-dex + IL-5-activated cells suggesting that IFN-γ induced IgG3 class switching. Of

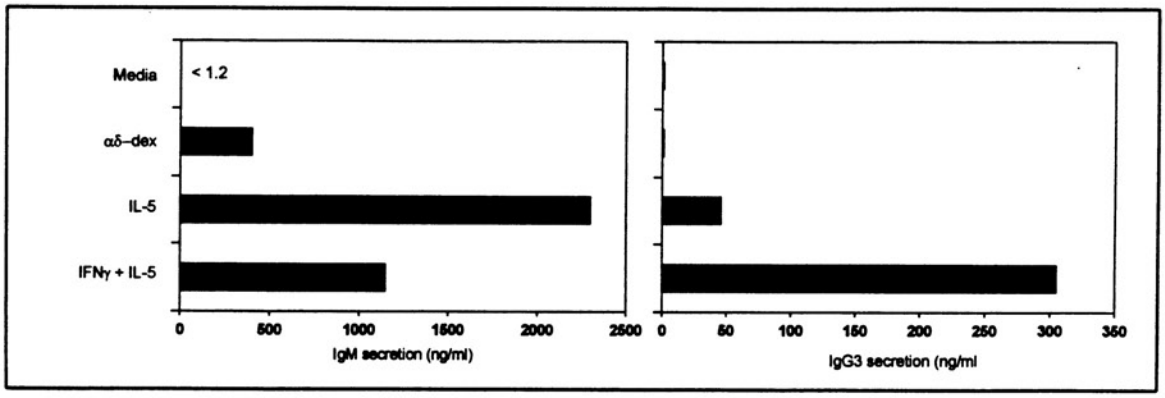


FIGURE 17. Effect of IFN-γ on IgG3 secretion by αδ-dex-activated B cells. Resting BALB/c B cells (final concentration, 1.25 x 10^5 cells/ml) were stimulated with αδ-dex (0.01 μg/ml) or LPS (20 mg/ml). IFN-γ (100 U/ml) was added to cultures as indicated. Culture supernatants were harvested on day 6 of culture and analyzed for IgM or IgG3 secretion by ELISA.

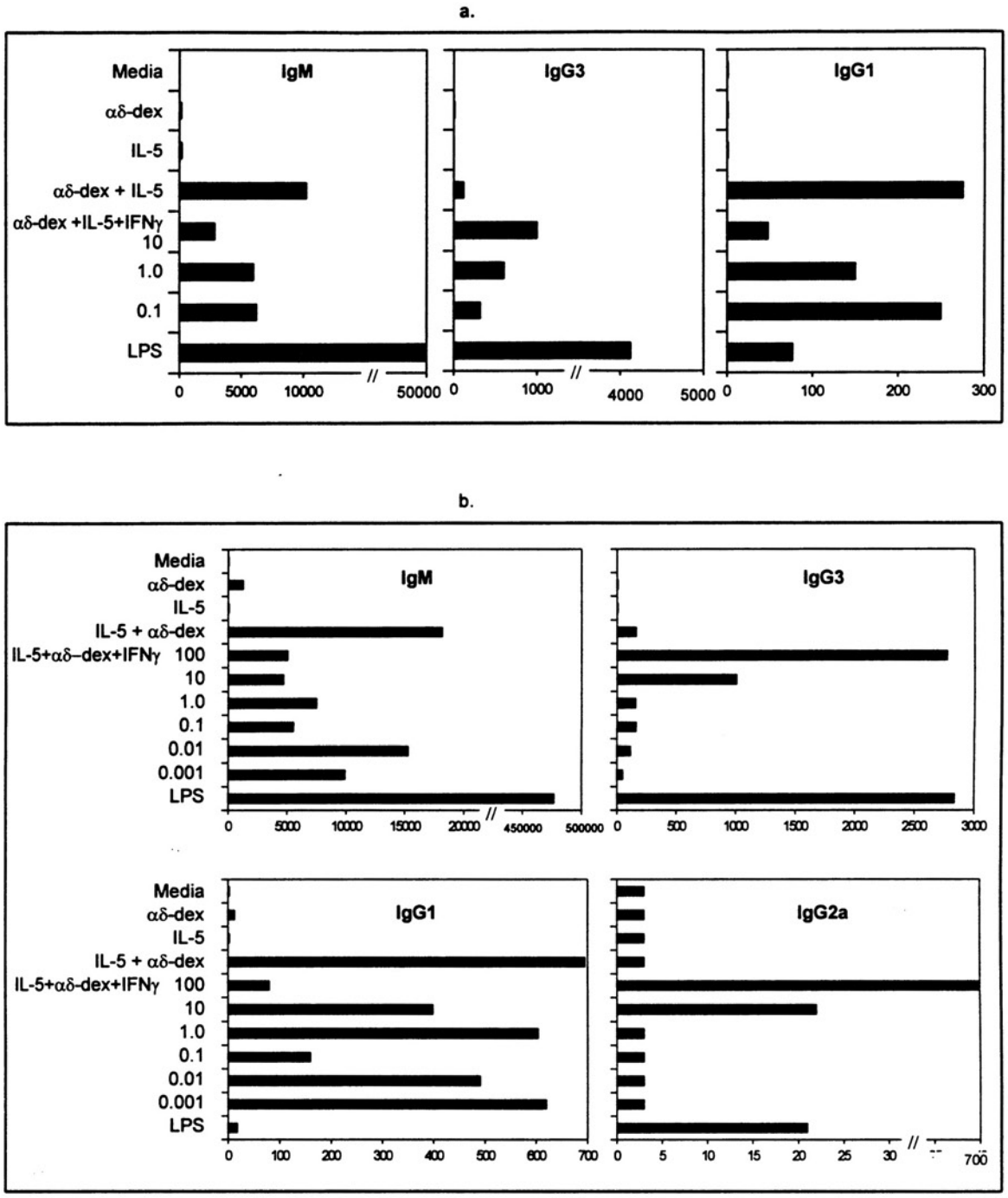


FIGURE 18. Determination of optimal concentration of IFN-γ for inducing IgG3 secretion by αδ-dex + IL-5-activated B cells. In two separate experiments (a, b), resting BALB/c B cells (final concentration, 1.5 x 10^5 cells/ml) were stimulated with LPS (50 μg/ml) or αδ-dex (3 ng/ml). αδ-dex-activated cells were analyzed in the presence or absence of IL-5 (150 U/ml), and log increments of IFN- γ, as indicated, were added to αδ-dex + IL-5-stimulated cells. Culture supernatants were harvested on day 6 of culture and analyzed for Ig secretion by ELISA.

interest, whereas IFN-γ was previously shown to induce IgG2a class switching by LPS-activated cells, it *inhibited* LPS-induced IgG3 class switching (260) thus, underscoring the critical role of the B cell activator in determining cytokine-mediated Ig isotype production. These data formed the basis for continued study by others in our laboratory to more definitvely demonstrate that IFN-γ was indeed an IgG3 switch factor and is summarized briefly as follows:

IFN-γ is a switch factor for the IgG3 subclass. Evidence to support the notion that IFN-γ induced IgG3 class switching first came from flow cytometry studies, using a FITC-labelled monoclonal anti-IgG3 antibody, which demonstrated that IFN-γ selectively increased the percentage of B cells expressing membrane (m)IgG3 after activation with αδ-dex + IL-5 (Figure 19). Thus, 4 days after initiation of culture, when Ig class switching is at its peak, B cells stimulated by αδ-dex + IL-5 in the presence of IFN-γ showed a >6-fold increase in the percentage of mIgG3$^+$ cells compared to that seen in the presence of αδ-dex + IL-5 alone. In contrast, IFN-γ had no significant effect on the small percentage of mIgG1$^+$ cells induced by αδ-dex + IL-5. The specificity of the FITC-anti-IgG3 mAb for binding mIgG3 was demonstrated by staining an aliquot of αδ-dex + IL-5 + IFN-γ-stimulated cells with FITC-anti-IgG3 mAb that had been preincubated with either an excess of myeloma mouse IgG3 or IgG1. IgG3, but not IgG1, bound to the FITC-labelled anti-IgG3 mAb and blocked binding of this mAb to the mIgG3$^+$ cells.

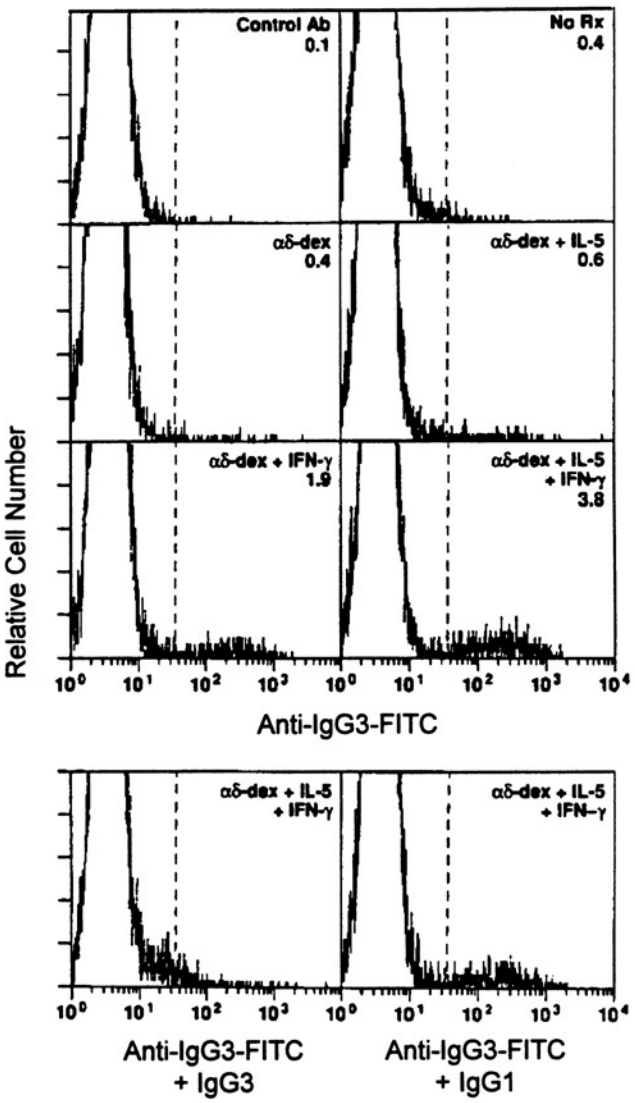


FIGURE 19. IFN-γ increases the percentage of mIgG3+ cells in αδ-dex + IL-5-containing B cell cultures. B cells were stimulated with αδ-dex (3 ng/ml) ± IL-5 (150 U/ml) ± IFN-γ (1 U/ml). Cultured cells were removed on day 4 for quantitation of mIgG3+ B cells by flow cytofluorometric analysis. 15,000 cells were analyzed fro each group. This data is representative of two similar experiments.

To determine whether IFN-γ directly stimulated B cells to synthesize IgG3 and to address whether it promoted this effect through induction of IgG3 class switching, resting, $mIgM^+mIgG3^-$ spleen cells were isolated by cell sorting and cultured in the presence of αδ-dex + IL-5 with or without IFN-γ (Table 10). IFN-γ stimulated a comparable increase in IgG3 secretion in both unsorted (>8-fold) and sorted (9-fold) cell populations while inhibiting the production of IgM and IgG1. Thus, IFN-γ was shown to act directly on $mIgM^+mIgG3^-$ B cells to stimulate IgG3 secretion.

Since an increase in steady-state levels of germline C_H RNA specific for a particular C_H gene typically precedes, and is thought to direct, class switching to the expression of that gene (135-137) studies were performed to determine whether IFN-γ selectively induced germline γ3 RNA in αδ-dex + IL-5-activated B cells. B cells were stimulated for 2 days with αδ-dex +/- IL-5 in the presence or absence of IFN-γ, and Northern blot analysis was performed using a cDNA specific for germline γ3 (Iγ3 probe) or germline γ1(Iγ1 probe) RNA. IFN-γ stimulated a significant increase in the steady-state level of germline γ3 RNA, while inhibiting germline γ1 RNA, by B cells stimulated with αδ-dex or αδ-dex + IL-5 (Figure 20).

SUMMARY

Collectively, the demonstration that IFN-γ increases the percentage of $mIgG3^+$ cells, stimulates IgG3 secretion by B cells which are $mIgM^+mIgG3^-$ at initiation of culture, and selectively

IFNγ Acts Directly on mIgM⁺mIgG3⁻ B Cells to Stimulate IgG3 Production

	Ig secretion		
	IgM	IgG3	IgG1
		ng/ml	
mIgM⁺ mIgG3⁻			
αδ-dex + IL-5	15,625	94	375
αδ-dex + IL-5 + IFNγ	10,000	850	33
Unsorted			
αδ-dex + IL-5	10,250	120	275
αδ-dex + IL-5 + IFNγ	2,875	1,000	48

TABLE 10. Resting BALB/c B cells were prepared and divided into two groups. One group was stained with FITC-anti-IgG3 + PE-anti-IgM and mIgM⁺mIgG3⁻ cells were isolated by electronic cell sorting. The second group of cells were not subjected to cell sorting. Cells from both groups were placed in cultured simultaneously at 1.25 X10^5 cells/ml in the presence of αδ-dex (3 ng/ml) and IL-5 (150 U/ml) alone or with IFN-γ (10 U/ml). Culture supernatants were removed 6 d after initiation of culture for measurement of Ig isotype concentrations by ELISA.

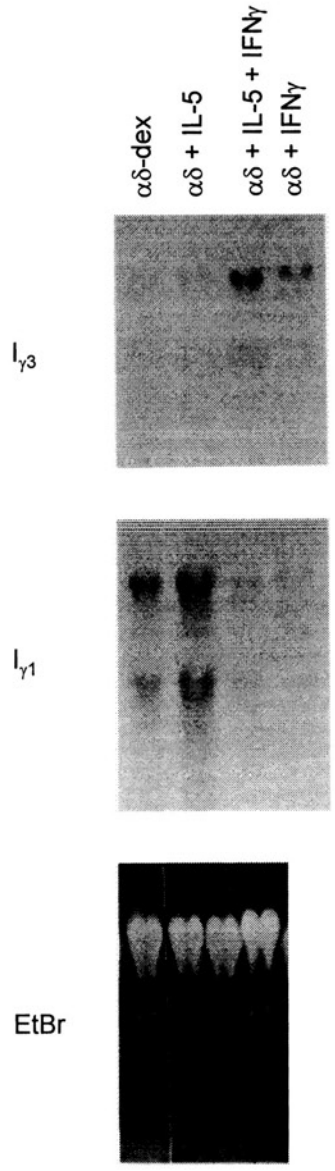


FIGURE 20. **IFN-γ induces germline γ3, but not γ1, RNA.** B cells were stimulated for 2 d with αδ-dex (3 ng/ml) ± IL-5 (150 U/ml) ± IFN-γ (1 U/ml). Total RNA was extracted and Northern blot analysis was performed using the indicated probes to detect CH-specific germline RNA. Ethidium bromide (EtBr) staining of the Northern blot indicates equal loading and transfer of RNA. Similar results were obtained in two additional experiments.

upregulates the levels of steady-state germline γ3 transcripts strongly suggests that, *in vitro*, IFN-γ, in the presence of IL-5, promotes IgG3 switch recombination by B cells activated with the TI-2-like antigen, αδ-dex.

Although no studies have determined the cellular basis by which TI-2 antigens elicit an *in vivo* IgG3 response, recent reports by other investigators have demonstrated a T-independent mechanism for IFN-γ production (319). In this regard, infection with the bacterium *Listeria monocytogenes* was shown to stimulate IFN-γ production by NK cells (319) and immunization of mice with the TI-2 antigen, TNP-Ficoll was shown to induce NK cell production of IFN-γ (320-322). Thus, in theory, infection with bacteria possessing TI-2 antigens could elicit T cell independent, antigen-specific IgG3 secretory responses through NK cell-derived IFN-γ acting on B cells activated, via their mIg signalling pathway, by the TI-2 antigen.

VI. GENERAL DISCUSSION AND FUTURE DIRECTIONS

These data add further support to the notion that Ig class switching is a highly regulated process. This has been shown to be true, in part, through the selective effects of distinct cytokines on transcriptional regulation, and hence accessibility, of Ig C_H genes for switch recombination (134-137), in the context of specific modes of B cell activation. LPS and αδ-dex have been used as TI-1 and TI-2 modes of B cell activation, respectively, whereas CD40L, which is induced on T cells upon their activation and stimulates B cells through their membrane CD40 molecule (285), has been used as a model for B cell activation in response to T cell-dependent antigens (284,285). These studies, along with studies by others in our laboratory (7,63,281,290,323-326), have helped to formulate the general notion that the B cell activator plays a determining, and not simply a permissive, role in cytokine-mediated Ig class switching and synthesis.

Thus the studies in this thesis show that: 1) whereas IFN-γ induces IgG3 secretion in αδ-dex-activated cells it is inhibitory for LPS-induced IgG3 switching, 2) IgG2b is induced by TGF-β in LPS-, but not αδ-dex-, activated B cells, and 3) while TGF-β is necessary for IgA class switching and induces small numbers of $mIgA^+$ cells upon activation with either LPS-, αδ-dex-, or CD40L-stimulated cells, the combined action of either LPS or CD40L with membrane Ig cross-linking, by αδ-dex, in the presence of IL-4, IL-5, and TGF-β is

required for the generation of relatively high percentages of $mIgA^+$ cells, comparable to that observed in the Peyer's patch.

Collectively, these and previous studies further showed that each of three cytokines can selectively regulate, in a positive manner, the class switch to two different Ig isotypes: IL-4 for IgG1 and IgE (1-5,13-15,17,88,90,136,137,147-149,303,304), IFN-γ for IgG3 and IgG2a (7,17,22), and TGF-β for IgG2b and IgA (23-26,281,324). These studies do not exclude the possibility that additional parameters exist that selectively regulate Ig isotype production. Thus, although Ig isotype regulation *in vivo* can frequently be accounted for, in part, by the action of known cytokine switch factors, it is clear that additional, unknown, pathways exist for inducing certain Ig isotypes after various immunizations. Further, it is often difficult, if not impossible to determine the nature of the activation signal(s) imparted to the responding B cells during an immune response, especially with antigenically complex microorganisms. Conclusions made regarding the antibody response following immunization with a defined antigen do not necessarily apply to all antigens of that biochemical classification. Thus, although immunization with polysaccharide antigens preferentially induce IgG3, studies have also demonstrated that some polysaccharides do not elicit production of this isotype such as alternative biochemical forms of dextran and polysaccharide purified from the capsule of *Neisseriae meningitus*. (327).

Nevertheless, our demonstration that IFN-γ induces IgG3 switching by αδ-dex-activated B cells provides a model system for evaluating the characteristic induction of IgG3 in response to immunization with TI-2 antigens. Thus, bacterial capsular antigens can activate NK cells, in a T cell-independent manner, which can then serve as a non-T cell source for IFN-γ (20,328). Indeed, the ability of polysaccharide antigens to fix complement through the alternative pathway (329) may allow for these antigens to interact with and stimulate a large number of immunoregulatory cells which bear complement receptors. Recent work in our lab has shown that supernatants from activated $CD4^+$ Th1 and Th2 clones and NK cells, in combination with IL-1 and IL-2, induce high rate Ig secretion by αδ-dex-activated, sort-purified B cells (325). GM-CSF, IL-3, and/or IFN-γ were later identified as the cytokines present in these supernatants that were responsible for this induction of Ig secretion (330). Since GM-CSF and IL-3, like IFN-γ, can also be secreted by non-T cells a means is provided not only for TI induction of class switching but for secretion of those Ig isotypes which are newly expressed.

LPS, like polysaccharide antigens, is also stimulatory for various other cell types in addition to functioning as a B cell mitogen. In this regard, LPS is a potent macrophage activation factor and stimulates the release of a variety of macrophage-derived cytokines including IL-1, IFN-γ, IL-6, PGE_2, and GM-CSF (331,332) While the direct proliferative activities of LPS on T cells is controversial, macrophage-derived cytokines can have significant effects on both T cells and NK cells and thus indirectly influence the humoral immune

response (331). Indeed the ability of LPS to induce IFN-γ *in vivo* could in theory account for the induction of IgG2a LPS-specific antibody titers following LPS immunization given that IFN-γ is an IgG2a switch factor for LPS-activated B cells *in vitro*.

A physiologic role for most murine Ig isotypes, based on their known, distinct effector function, and the contexts in which they are induced, has been suggested. Although IgG2b is induced in several bacterial and viral infections, its role in the humoral immune response is particularly mysterious, and until recently no specific model system has been developed to study the function of this isotype. However, Zhang et al. recently generated somatic chimeric mice in which splenic B cells have a selective defect in IgG2b class switching (333). These mice were generated by replacing the Iγ2 b promoter and I exon of embryonic stem (ES) cells with a neomycin resistance gene. The mutated ES cells were then injected into RAG-2 deficient blastocysts. Since RAG-2 -/- cells cannot generate mature lymphocytes, all lymphocytes were ES-cell derived. Mice that were homozygous for the mutation were deficient in IgG2b production and in the expression of germline γ2b transcripts resulting in a specific defect in class switch recombination to Sγ2b sequences. Mice heterozygous for the mutation demonstrated a partial decrease in their ability to switch to γ2b. While these results clearly demonstrate that the I region is important in regulating switch recombination, this system did not knockout all production of IgG2b and thus merely reduced the efficiency of recombination (J. Zhang, personal communication). Although these mice were not analyzed

for further phenotypic changes in this study, additional knowledge regarding the molecular regulation of IgG2b and the physiologic necessity for this isotype could be accomplished using this system.

Our demonstration that TGF-β functions as an IgG2b-switch factor in LPS-activated B cells will also enable further analysis of the regulation of IgG2b production at both the molecular and cellular level following specific antigenic challenge. In this effort, I attempted to establish a physiologic role for TGF-β in IgG2b class switching through *in vivo* experiments. This work demonstrated that an LPS-specific IgG2b response can be elicited in BALB/c mice following LPS immunization. However, further investigation to link antigen-specific IgG2b production with endogenous TGF-β was unsuccessful since immunization with an anti-TGF-β mAb did not abolish the LPS-specific IgG2b response. Insufficient neutralization of endogenous TGF-β may explain this inability to establish an *in vivo* correlation to the *in vitro*-demonstrated role for TGF-β in IgG2b class switching. It is likely that systemic injections of high concentrations of anti-TGF-β mAb were insufficient to neutralize all endogenous TGF-β present. In this regard, TGF-β message has been shown to be constitutively present in many different cell types (188). Although much of the endogenous TGF-β protein exists associated with latency-associated peptide (LAP), studies have demonstrated that mature TGF-β can be reversibly dissociated from and reassociated with LAP thus resulting in the gain and loss of biological activity (334). This mechanism may allow for reserve stores of TGF-β which escape neutralization by the anti-TGF-β mAb

used. Furthermore, it has been documented that inhibited production of TGF-β by a particular cell type results in enhanced TGF-β production by alternative cell types (335). In this regard, treatment with anti-TGF-β could evoke a compensatory enhancement in TGF-β production by various TGF-β-producing cells.

To follow up on these initial findings, several preliminary experiments were performed in an effort to further correlate TGF-β with IgG2b class switching. While each of these experiments had specific complications and thus were not previously discussed in this thesis, a brief discussion of the experimental questions asked, the experimental design used, and the complications encountered will provide insight for the direction of future studies evaluating the role of TGF-β in IgG2b class switching.

Initial preliminary studies evaluated whether the *in vivo* generation of mIgG2b$^+$ cells is TGF-β-dependent. In these experiments, spleen, peritoneal, and Peyer's patch B cells were isolated from unimmunized and LPS-immunized BALB/c mice. These cells were then analyzed by flow cytometry for the percentage of B cells expressing various membrane Ig isotypes. No significant induction of mIgG2b was observed in LPS-immunized mice compared to saline-injected control mice. Similar results were obtained for various concentrations of LPS analyzed. Thus, while LPS immunization enhanced the serum level of LPS-specific IgG2b, no enhancement in mIgG2b+ cells could be detected in either spleen, peritoneal cavity, or Peyer's patches. Therefore these preliminary

studies suggested that intraveneous immunization with LPS does not induce sufficient enhancements in mIgG2b$^+$ cells to be used for monitoring TGF-β-regulated changes in IgG2b. The failure for mIgG2b expression to be upregulated is not surprising. In contrast to the serum LPS-isotype specific ELISA, the flow cytometric analysis of mIgG2b$^+$ cells following LPS immunization does not merely detect LPS-specific mIgG2b$^+$ cells but, instead, relects the change in total mIgG2b expression. Hence, changes in LPS-specific mIgG2b$^+$ cells may be masked by concomitant changes in specific mIgG2b expression for additional antigens.

Further preliminary studies focused on determining whether a role for endogenous TGF-β in the LPS-induced enhancement of serum IgG2b could be demonstrated through the combined administration of anti-TGF-β and elimination of macrophages, a primary cell source of endogenous TGF-β. Van Rooijen has shown that intravenous adminstration of liposome-encapsulated dichloromethylene-diphosphonate eliminates macrophages in spleen and liver (336,337). Liposomes are ingested by macrophages which are then destroyed following phospholipase-mediated disruption of the liposomal bilayers and release of the toxic drug. These experiments were based on the hypothesis that elimination or reduction of macrophages would diminsh the level of endogenous TGF-β. Thus, this would allow the concentrations of anti-TGF-β administered to neutralize sufficient amounts of endogenous TGF-β resulting in levels which are insufficient to induce *in vivo* IgG2b class switching. Results of preliminary experiments suggested that neither liposomes

alone or in combination with anti-TGF-β mAb would reduce the level of serum IgG2b in LPS-immunized mice compared with those receiving LPS only. These results must be regarded with caution since small numbers of mice were analyzed. Furthermore, assessment of macrophage depletion was performed by flow cytometrically analyzing the reduction in $F4.80^+$ cells, a macrophage cell surface marker. While a modest reduction in macrophages was observed, this was accompanied by a 2-fold decrease in T cells and in NK cells. Thus the inability to diminish serum IgG2b levels may be due to ineffective macrophage elimination in combination with the already discussed ineffective neutralization by anti-TGF-β mAb. The concommitant decrease in additional cell types, however, is beneficial in that it serves to reduce additional endogenous sources of TGF-β.

Additional studies were performed to correlate levels of serum TGF-β with serum IgG2b levels following LPS immunization and to evaluate the effectiveness of serum TGF-β neutralization following treatment with anti-TGF-β mAb. Since TGF-β exists in a latent and active form (203), TGF-β levels were analyzed by bioassay rather than ELISA which detects both forms of TGF-β. This enabled a direct assessment of the level of active TGF-β and an indirect assessment of the level of latent TGF-β following conversion of the latent to the active form by heat activation. These preliminary studies suggested that serum TGF-β levels were approximately 4-fold greater in LPS immunized animals compared with saline-injected control animals. Following serial 2-fold dilution of serum obtained from control or LPS-immunized mice, maximal DNA synthesis of HT-2 cells employed

in this bioassay, which are growth-inhibited by TGF-β (338), was achieved at lower serum dilutions in the presence of anti-TGF-β compared to that observed in the absence of anti-TGF-β. Although no analysis was performed on undiluted serum, the results of this preliminary study showed that HT-2 DNA synthesis was still greatly suppressed in the presence of serum diluted 1:10 following the addition of 30 μg/ml of anti-TGF-β. This suggested that significant levels of active TGF-β were not neutralized by the antibody. A comparison of HT-2 DNA synthesis for all serum dilutions in the presence and absence of anti-TGF-β showed that maximal DNA synthesis could be achieved by serum treated with anti-TGF-β at significantly lower serum dilutions than that observed for serum in the absence of anti-TGF-β.

The results of this *in vitro* analysis could correspond to the inability to sufficiently neutralize endogenous TGF-β. For *in vivo* studies, 2 mg of anti-TGF-β mAb was injected at two different time points for a total of 4 mg of antibody administered per mouse. We calculated that this would be a sufficient dose of antibody for *in vivo* neutralization, since the typical blood volume per mouse is approximately 3 ml. Thus a concentration of 667 μg/ml was administered to each mouse per injection, however, this may not have been sufficient for endogenous TGF-β neutralization.

These results must be interpreted with caution since in the process of blood coagulation, platelets aggregate and are a primary source of TGF-β in serum (188). Thus these results, while

demonstrating differences in serum TGF-β levels in control and LPS immunized mice, as well as the ability for anti-TGF-β to neutralize TGF-β present in serum, do not solely reflect LPS-induced changes in endogenous TGF-β levels. To more definitively demonstrate quantitative differences, future determinations should be accomplished on plasma samples which would not contain platelet-derived TGF-β.

A series of preliminary studies were also performed to follow up on the observation that DBA/2-derived B cells consistently secrete significantly higher levels of IgG2b following LPS stimulation compared with BALB/c mice. Analysis of IgG2b secretion following LPS activation of splenic B cells in 9 different murine strains (by the excellent technical assistance provided by M. Lugo) demonstrated that they segregated into high and low IgG2b producers: 4 strains (DBA/2, C3H, C57Bl/6, and CBA/J) secreted higher levels of IgG2b, 3 strains (BALB/c, AKR, and A/J) secreted comparatively lower levels of IgG2b, and the 2 remaining strains (C3H/HEJ and C57Bl/10) were excluded since they secreted minimal IgG2b but also demonstrated suppressed secretion of IgM and IgG3.

Subsequently, I performed a preliminary experiment to evaluate the levels of endogenous TGF-β produced by LPS-activated B cells from each of the 9 murine strains previously analyzed for IgG2b secretion. Supernatants from LPS-activated B cell cultures of each strain were analyzed by TGF-β bioassay following heat-activation for total levels of TGF-β, including both latent and active

forms, and for the levels of active TGF-β in the absence of heat-activation. These studies were unsuccessful since DNA synthesis of the TGF-β sensitive HT-2 cells, in the presence of undiluted, non-heat-activated supernatant from each strain, was equal to the maximal level of HT-2 DNA synthesis in the absence of supernatant. This could indicate that either the sensitivity of this bioassay is not sufficient to detect low levels of active TGF-β present in the supernatant or that no active TGF-β was present in any of the supernatants. Undiluted, heat-activated supernatants from the various strains did, however, variably inhibit [^{3}H]-TdR incorporation by HT-2 cells, thus indicating that conversion of latent to active TGF-β resulted in levels of active TGF-β that were detectable by the bioassay. Although the various strains exhibited modest differences in the levels of total TGF-β, they did not appear to segregate into the same groups as those identified for IgG2b secretion. This quantitation of total TGF-β, however, is not indicative of the level of biologically active TGF-β present in the supernatants. Although this experiment should be repeated to assess it's validity, these data suggest that it may not be possible to measure TGF-β in LPS-activated B cell supernatants using this bioassay.

One precautionary note with regard to the previous analysis is that Burger et al. have reported that fetal calf serum (FCS) contains concentrations of TGF-β which vary considerably between different lots (339). Our assays were performed with FCS that we demonstrated to be optimal following screening of various lots. This determination was made by culturing LPS-activated BALB/c B cells

in FCS-containing medium and selecting the lot of FCS which produced relatively low levels of secreted IgG2b. Although the HT-2 cells are maintained in reduced concentrations of FCS (4%) throughout the bioassay, the culture supernatants contain 10% FCS. To completely eliminate TGF-β from the FCS it could be passed over an anti-TGF-β affinity column. However, this procedure may not be necessary for these determinations if non-heat-activated, undiluted supernatants reproducibly result in maximal HT-2 DNA synthesis thus indicating TGF-β levels are below the limits of detection.

To evaluate if strain differences exist at the TGF-β transcriptional level, the amount of TGF-β mRNA produced by LPS-activated B cells, was quantitated by RT-PCR for five mice in each of two groups. One group represented a high IgG2b producer (DBA/2) and another represented a low IgG2b producer (BALB/c). The results demonstrated that approximately equal levels of TGF-β mRNA were produced by DBA/2 and BALB/c mice following LPS stimulation and thus, these strains did not appear to segregate, with regard to TGF-β mRNA levels, in a similar manner to that observed for IgG2b secretion. Since this preliminary experiment did not include a comparative analysis of non-LPS stimulated B cells from each strain, the contribution of LPS stimulation toward the production of TGF-β mRNA cannot be evaluated. Furthermore, upon including this control, the data would have to be interpreted with caution since the amount of TGF-β mRNA does not necessarily correspond to the level of biologically active TGF-β.

To summarize our *in vivo* IgG2b studies, we attempted to determine the physiologic relevance of the *in vitro*-established role for TGF-β in IgG2b class switching by three approaches: 1) by analyzing whether the *in vivo* LPS-induced generation of mIgG2b$_+$ cells is TGF-β dependent, 2) by analyzing whether serum elevations of IgG2b upon LPS immunization is dependent upon the release of endogenous TGF-β, and 3) by analyzing whether changes in serum IgG2b could be correlated with changes in the level of endogenously produced TGF-β. As mentioned, several complications impeded these determinations. The most significant of these complications can be attributed to: 1) the ubiquitous presence of TGF-β, as a result of it's *in vivo* production by many different cell types and it's secretion by activated platelets in serum test samples as well as FCS added to culture medium, 2) the inability to sufficiently neutralize *in vivo*-produced TGF-β, and 3) the apparent inability to detect low levels of TGF-β following in vitro LPS-stimulation. By incorporating the modifications previously mentioned, several of these methods could be used to define the physiologic role of TGF-β in IgG2b class switching. Further insight may also be obtained by performing these studies on mice in which the Iγ2b region has been genetically altered. Additionally, the identification of a means to generate a localized IgG2b response rather than a systemic response, such as that generated by intravenous LPS immunization, might alleviate the necessity for neutralizing all endogenous TGF-β.

Recently 3 phenotypic forms of IgG2b have been demonstrated to be due to alternative O-glycosylation at a specific amino acid in

the hinge region of one or both heavy chains (340). As a result, the various IgG2b forms demonstrate differential sensitivity to protease treatment with the resultant formation of different cleavage products. In particular, the univalent Fab/c cleavage product of the IgG2b form which possesses asymmetric H chain O-glycosylation has received particular attention due to enhanced capacity for tumor cell killing in comparison to its bivalent counterpart (341). Furthermore, a conserved glycosylation site in the C_H2 domain of both H chains demonstrates heterogeneity in the level of glycosylation which is dependent on several factors including pathological state and culture conditions of IgG2b producing cells (340). Again this heterogeneity could lead to differential susceptibility to protease digestion which can have significant impact on the ability to perform effector functions such as complement activation and induction of ADCC mediated through the binding of Fc receptors present on macrophages (342-344). In an effort to identify a means to generate a localized IgG2b response, knowledge of these alternative IgG2b forms and the factors that regulate their expression may identify specific phenotypic forms better suited for specific microenvironments.

Additional assessment of the transcriptional regulators for both TGF-β and IgG2b production may also enhance our understanding of the mechanisms regulating production of this Ig isotype. In this regard, a recent study suggested a correlation between c-*fos* expression and IgG2b production by LPS stimulated B cells from a transgenic mouse carrying the mouse c-*fos* gene under the control of

an interferon α/β inducible promoter (345). In the presence of prolonged high c-*fos* expression, IgG2b production was suppressed however, no suppression in IgG2b production was present following early, low level c-*fos* expression. Elevated levels of c-*fos* mRNA were concomitantly present with enhanced levels of AP-1 nuclear proteins (Jun/Fos complex). Further analysis of these findings may provide interesting insight to the mechanisms by which TGF-β regulates the IgG2b class switch since AP-1 binding sites have been implicated in the transcriptional control of TGF-β1 expression.

While the IgG3 and IgG2b studies presented in this thesis were focused on elucidating a specific cytokine that regulates each class switching event, our IgA studies were directed at determining the conditions required for optimal *in vitro* IgA class switching. Although TFG-β had previously been shown to upregulate germline α transcripts and IgA secretion by LPS-activated B cells (23,26,282), the percentage of mIgA$^+$ cells obtained *in vitro* with this stimulation was considerably lower than that observed in Peyer's patches (28). Thus it was concluded that several factors, in addition to TGF-β, were required for *in vivo* class switching.

Regardless of the mechanism, these data suggest a rather stringent, complex requirement for inducing high percentages of mIgA$^+$ cells under physiologic conditions. The fact that splenic B cells, as used in these studies, can undergo vigorous IgA class switching suggests that the pattern of stimulation, and not simply the intrinsic nature of the B cell population, plays an important role in

this phenomenon. Although any of the IgA-inductive stimuli identified in this report could become manifest in any immunologic site, the sequestration of IgA class switching within the Peyer's patch could, in part, reflect the constitutive and simultaneous action of high levels of multiple stimuli on the resident B cells, as a result of their unique proximity to and communication with the complex microbial and antigenic milieu of the gastrointestinal tract. In this regard, Peyer's patches have three anatomically as well as functionally distinct regions which may enable this unique interaction (27,28,346): 1) The dome region is covered by an M cell-containing epithelium which makes initial contact with antigen present in the gastrointestinal tract and is enriched for B and T lymphocytes and macrophages. 2) The follicular region contains germinal centers and is the primary region for active B cell division. This region contains an increased proportion of $mIgA^+$ cells and is thought to be the major site where B cells are initially commited to IgA. 3) The parafollicular region predominantly contains T cells which are thought to support IgA responses by their secretion of various cytokines. Thus, the concommitant exposure to multiple proteins with consequent recruitment of T cell help, as well as exposure to T-independent antigens, including mitogens and polysaccharides which could deliver multivalent antigen-receptor-mediated signals, could allow for vigorous IgA class switching to occur in response to TGF-β.

The inductive effects of IL-4 and IL-5 and the inhibitory effects of IFN-γ for IgA class switching further suggests that the Th subset dichotomy (91) is relevant to understanding the regulation of

IgA class switching *in vivo*. In this regard, while Peyer's patches are considered to be IgA inductive regions, lamina propria is considered to be an IgA effector region (347). This distinction refers to the lack of final B cell differentiation into IgA-secreting plasma cells within the Peyer's patches and the requirement for $mIgA^+$ B cells to travel to distant mucosal sites for this maturation process (347). Since IL-4 and IL-5 have been reported to be produced in both anatomical sites (347,351), our demonstration that stimulation with these two cytokines, in the presence of TGF-β and dual B cell activation, results in increased $mIgA^+$ cells as well as enhanced IgA secretion suggests that additional mechanisms exist within these sites to regulate the *in vivo* differentiation to IgA plasma cells. Thus the communication between Th1 and Th2 lymphocytes via the cytokines they secrete may be a crucial factor for regulating IgA differentiation. Recent studies by Taguchi et al. demonstrate that the ratio of IL-5-producing T cells (Th2) to IFN-γ-producing T cells (Th1) in lamina propria was 3:1 compared to approximately equal (low) levels observed in Peyer's patches (347). Previous studies have demonstrated a cross-regulatory role for cytokines secreted by Th1 and Th2 cells. (349,350) For example, IFN-γ produced by Th1 cells down-regulates the function of Th2 cells and IL-10 secreted by Th2 cells inhibits Th1 cells. Although no comparison of additional cytokine levels (IL-2, IL-4, IL-6) was made in the studies by Taguchi et al., they suggested that specific ratios of Th1 and Th2 cells may be necessary in both IgA inductive and effector sites for homeostasis of mucosal immune responses (347).

The use of the *in vitro* IgA switching system described in this thesis should provide a means to evaluate several unanswered questions regarding IgA induction that may point towards *in vivo* mechanisms. In particular, the ability for other cytokines to replace either IL-4 or IL-5 in this system, or to inhibit or further augment levels of $mIgA^+$ cells will expand our understanding of the potential pathways that could be employed normally *in vivo* and/or point to mucosal vaccination strategies for maximizing a protective IgA secretory response. Our demonstration that IL-2 can substitute for IL-5 in stimulating IgA class switching by B cells activated by LPS/$\alpha\delta$-dex but not CD40L/$\alpha\delta$-dex in the presence of TGF-β + IL-4 suggests that the particular mode of B cell activation may play a critical role in IgA class switching by influencing the specific cytokine distribution or by augmenting B cell responsiveness (by upregulating specific cytokine receptors) to specific cytokines.

The physiolgoic implications of the IL-10-mediated inhibition of LPS-driven IgA switching requires further study. One possible implication of this effect lies in the ability of LPS itself to induce IL-10 secretion in B cell-enriched spleen cell populations (J.J. Mond, ongoing studies). Thus, IL-10 could be part of an autocrine loop for limiting IgA class switching. Interestingly, mice made genetically deficient for IL-10 production demonstrated slightly elevated levels of antigen-specific IgA following immunization with the TD antigen haptenated chicken γ-globulin than those observed for normal IL-10-producing counterparts (293). In contrast, anti-IL-10 treated mice demonstrated significantly lower levels of serum IgA (352). While

the reason for this difference is not understood, these studies may not have direct relevance since serum IgA is predominately bone marrow- and not MALT-derived (27,28,346), however, it further emphasizes the significance of IL-10 in the generation of IgA responses.

Studies investigating whether additional cytokines can be substituted for IL-4 in this system are also warranted. While our studies did not indicate the ability for either IL-2 or IL-10 to replace IL-4 for the generation of $mIgA^+$ cells, it is likely that another cytokine may function in this capacity since mice made genetically deficient for IL-4 remain capable of generating IgA responses (89).

IL-6 is known to enhance Ig secretion by activated B cells (356-359) and has been implicated in stimulating IgA secretory responses, although it is not directly involved in the process of switch recombination (354,355). In this thesis we demonstrated a direct correlation between the generation of $mIgA^+$ cells and secretion of IgA in response to the stimuli that are included our IgA induction system. Hence, significant induction of $mIgA^+$ cells occurred in the absence of IL-6. In subsequent studies in our lab, using B cells from IL-6 knockout mice, no critical role for endogenous IL-6 in the induction of IgA secretion was observed: B cells from IL-6 knockout mice secreted IgA *in vitro* to a comparable degree relative to B cells obtained from wild type control mice. In contrast, IL-6 knockout mice were previously shown to have reduced levels of mucosal IgA-secreting cells *in vivo* and produce deficient local IgA

antibody responses following intranasal challenge with ovalbumin or vaccinia virus (360,361). Thus, *in vivo,* IL-6 may be an important cytokine in regulating the synthesis of secretory IgA.

Our studies also suggest that IFN-γ may play a key inhibitory role in the generation of IgA responses in Peyer's patches. In our studies, IFN-γ at concentrations as low as 1-10 U/ml were found to be inhibitory for the generation of $mIgA^+$ cells. Of interest, however, mice which were made genetically deficient for the expression of the IFN-γ receptor had reduced antigen-specific <u>serum</u> IgA titers in response to challenge with an attenuated rabies virus, relative to the wild type control mice (353). In this study Peyer's patches were not studied to assess the relative percentages of $mIgA^+$ cells nor were the relative numbers of IgA-secreting cells in the lamina propria determined. Furthermore, our lab has recently demonstrated that IFN-γ can strongly stimulate activated B cells to secrete Ig *in vitro* in an isotype-non-specific manner (325). As indicated above, one must be careful in comparing serum IgA responses with those that occur locally at the mucosa since the regulatory pathways for these two events may be distinct (27,28,346). The parameters which regulate IgA class switching (generation of $mIgA^+$ cells) and maturation of $mIgA^+$ cells into antibody secretors may differ both in the cytokines and/or other activators involved as well as the particular microenvironments in which these processes occur (362).

In addition to analyzing the capacity for alternative cytokines to augment or replace the activity of the cytokines employed in our

system for high-rate IgA class switching, the ability for additional modes of B cell activation, including T cell membranes or additional T cell surface molecules, to stimulate high level IgA induction could be determined. This may provide insight into the *in vivo* IgA regulatory mechanisms provided by various antigenic stimuli.

The molecular basis for this multiparameter requirement to generate mIgA$^+$ cells needs to be determined. Thus, the effect of each stimulatory component on the regulation of the steady-state levels of germline α RNA (i.e. $C_H\alpha$ "accessibility") would shed light on the underlying mechanisms which generate an IgA class switch. Changes in steady state germline α transcripts in response to a particular stimulus, would lead to studies to determine whether this is due to an increase in the transcriptional rate at the $C_H\alpha$ locus or due to enhanced stabilization of germline $C_H\alpha$ mRNA. Nuclear run-on assays could be used to assess transcriptional rate, and measurement of mRNA stability could be made by determining germline $C_H\alpha$ RNA half life.

Although induction of mIgA$^+$ cells suggests an underlying induction of $C_H\alpha$ gene rearrangement this, in theory, may not always be the case and thus needs to be directly assessed in a particular system. Direct measurment of $C_H\alpha$ DNA rearrangement could be made using the newly developed technique, digestion-circularization polymerase chain reaction (DC-PCR) (363) which would enable detection and quantitation of switch (S)μ-Sα recombination at the DNA level. Additionally, electrophoretic-mobility shift assays

(EMSA) could be used to evaluate the role of DNA binding proteins, which could in theory bind at a number of distinct loci in the Ig locus, in the regulation of IgA switch recombination.

In this regard, mice made genetically deficient in the expression of a 3' α enhancer show defective switching from IgM to IgG3, IgG2b, IgG2a, and IgE, but appear to switch normally to IgG1 and IgA suggesting that perhaps another enhancer is involved in IgG1 and IgA switching (157). In addition, recent work in our laboratory, in collaboration with Dr. David Baltimore, has indicated that B cells from mice made genetically deficient in the p50 component of NF-κB, a pleiotropic transcription factor, while capable of undergoing significant IgG1 switching, are markedly deficient in switching to IgA, IgE, or IgG3. Further studies are warranted to further understand the events that are occurring at the molecular level.

The demonstration that sorted $mIgM^{+}mIgA^{-}$ splenic B cells generated lower percentages of mIgA+ cells, relative to non-sorted, T cell-depleted, small B cell-enriched spleen cells after multiparameter stimulation for high-rate IgA induction requires further study. There are at least several hypotheses to account for this: (1) cell sorting has removed a population of non-B, non-T cells which are required for optimal IgA class switching, (2) a large proportion of the $mIgA^{+}$ cells generated in this system are arising from a small population of $mIgA^{+}$ cells present in the starting population. Since anti-IgD-dextran is necessary to drive this system, one would have

to entertain the unlikely possibility that these mIgA$^+$ cells also express mIgD, (3) the diminished generation of mIgA$^+$ cells is an artifact of cell sorting, i.e. that the sorting process makes the cells less vigorous functionally and thus unable to respond as well to the inducing stimuli.

These hypotheses can be tested experimentally by at least two methods. (1) Non-B, non-T cells (including macrophages, dendritic cells, and NK cells) could be separately deleted from the sorted mIgM$^+$mIgA$^-$ population. Additional studies could be performed in which the various cell types are independently added back to the spleen cell population. If deletion of a particular cell type abolished the high level of IgA induction and readdition of the cells reconstituted the high levels this would definitively show the necessity for these cells in this system. (2) B cells can be sorted, irrespective of membrane Ig isotype, to determine whether this causes a reduction in IgA class switching. In this latter case both mIgM$^+$ and any mIgA$^+$ cells would be included in the starting population. Further analysis of IgA class switching in these populations in the presence or absence of sorted non-B, non-T cells would be carried out.

This identification of a system for generating high levels of mIgA$^+$ cells *in vitro* provides a means to further investigate the regulatory mechanisms which govern mucosal immune responses. A more thorough understanding of these mechanisms, both in the gastrointestinal region as well as other mucosal-associated lymphoid

tissues, bears considerable clinical significance since knowledge of the elements which are involved in activating mucosal immunity allows for manipulation of the system. In particular, this would be valuable for understanding and treating a variety of diseases restricted to mucosal regions and for developing more efficient oral vaccines targeted at mucosal surfaces where many organisms gain their entry.

Several approaches to developing vaccines that are aimed at boosting mucosal immunity are currently being used or investigated. One example is the design of antigen carriers which are targeted to mucosal lymphoid tissues (364,365). *Vibrio cholerae*, which colonizes the GI tract and is engulfed by Peyer's patch M cells, and in which the toxin-encoding genes have been deleted, has been a strong candidate for use as a GI-targeted antigen carrier (365). In this manner, attenuated *V. cholerae* has been proposed for use as a shuttle of DNA segments of organisms not endemic to the GI tract to sites where mucosal responses could be triggered. Along these same lines, recombinant Bacille Calmette-Guerin (BCG) is being used to deliver proteins from the organisms which cause Lyme disease, tuberculosis, and urinary tract infections (365) For certain diseases such as Lyme disease, studies in mice demonstrated that this carrier was effective in delivering protection against subsequent challenge when delivered intranasally. The use of this vehicle as an antigen carrier has proven to have increased effectiveness due to its ability to generate a serum IgG antibody response, which more effectively

combats rechallenge with the pathogen, in addition to generating a strong mucosal IgA response.

Vaccines targeted to mucosal regions and delivered either orally or intranasally are exposed to the increased acidity of the stomach which can greatly decrease their effectiveness. Several approaches to shield antigens from these host defenses are being used including encapsulating the antigen in microspheres (364,365). When liposomes are used, the potential for vector-triggered immune responses are eliminated, however, the size of the encapsulated antigen can affect its ability to be engulfed by mucosal lymphoid cells such as Peyer's patch M cells.

Although each of these methods for delivering antigen to mucosal regions have been successful in generating immediate protective immune responses, the generation of permanent protection through memory responses has been less successful (365). In this regard, a more thorough understanding of the mechanisms which regulate mucosal immunity may provide insight for stimulating enhanced memory cell generation.

In summary, the studies performed in this thesis have identified regulatory factors involved in IgA, IgG2b, and IgG3 class switching and have provided a basis for additional investigation of further regulatory mechanisms at both the cellular and molecular level. An understanding of the systems that regulate class switching to these as well as other Ig isotypes has significance not only for

treatment of pathologic states in which these isotypes are involved but also in the development of effective vaccine strategies for boosting specific immunologic responses against specific pathogenic and antigenic stimuli.

VII. APPENDICES

Appendix A

Induction of Immunoglobulin Isotype Expression by Combinations of Cytokines and Permissive Stimuli

	Ig isotype expression in mice		
	Permissive stimuli		
Cytokine	LPS	T cell help	Anti-IgD-Dextran
IL-4	IgG1, IgE	IgG1, IgE	IgG1
IFN-γ	IgG2a	IgG2a, IgG3	IgG2a, IgG3
TGF-β	IgA, IgG2b	IgA, ?IgG2b	IgA

Table 11. Induction of specific Ig class switching in the presence of specific cytokines in combination with T-dependent (T cells), T-independent type 1 (LPS) and T-independent type 2 (Anti-IgD-dextran) modes of B cell activation. (Adapted from reference 41, used with permission.)

Appendix B

Murine Ig Heavy Chain Gene Locus

Chromosome 12

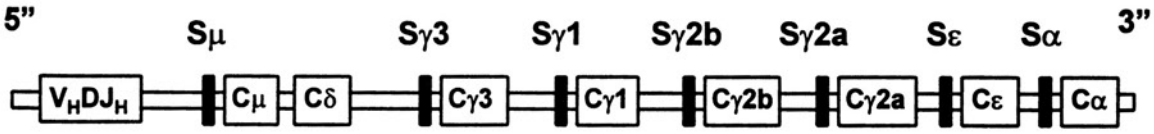


Figure 21. The murine C_H locus is located on Chromosome 12, 3' of the rearranged V_HDJ_H gene which encodes antigen specificity and is comprised of eight distinct C_H genes which determine Ig isotype. This locus spans approximately 200 Kb with the C_H genes appearing in the sequence configuration depicted above.

Appendix C

Looping Out and Deletion

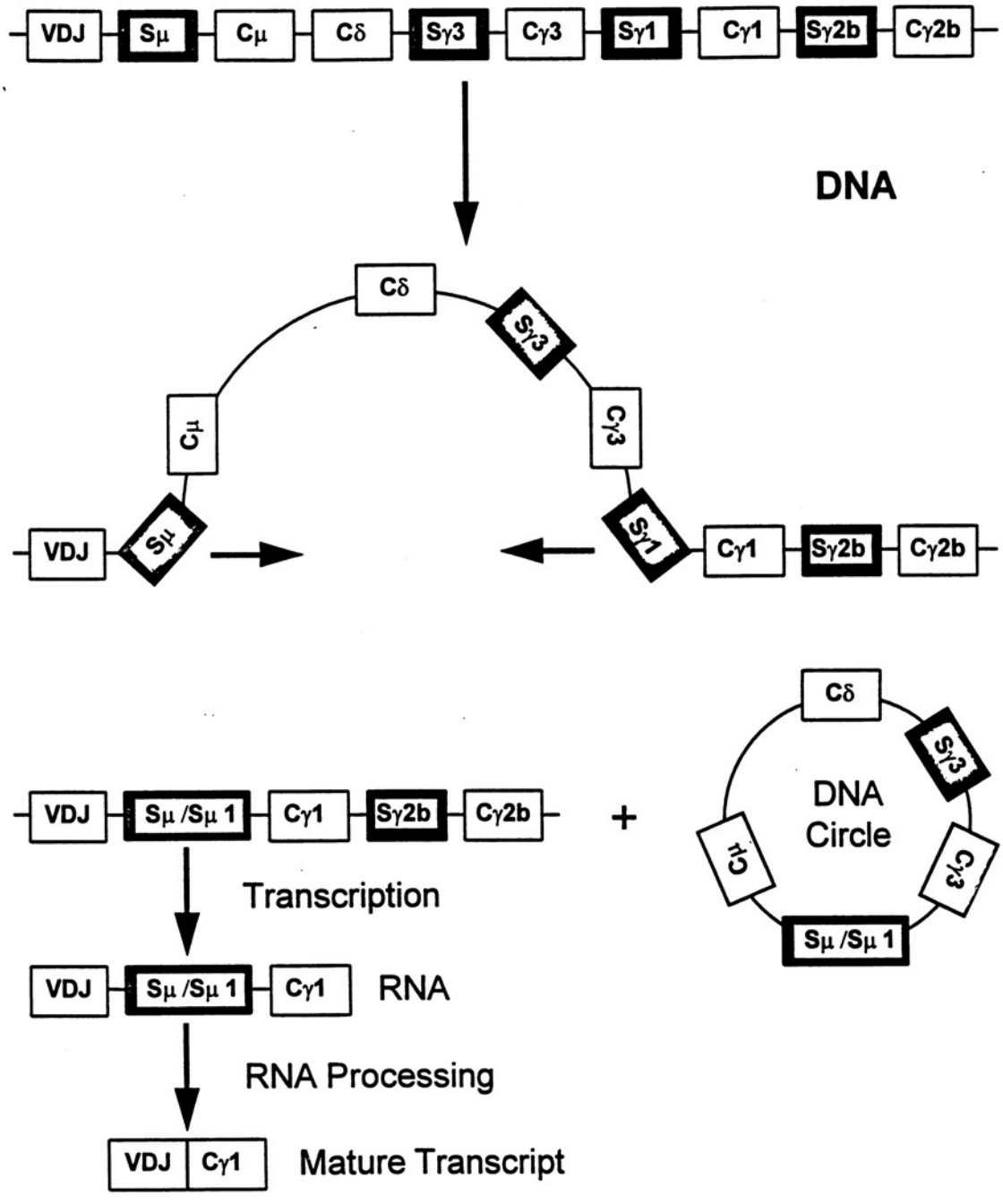


Figure 22. Recombination between two sites on the same DNA segment with looping out and excision of the intervening DNA. (Used with permission, reference 41.)

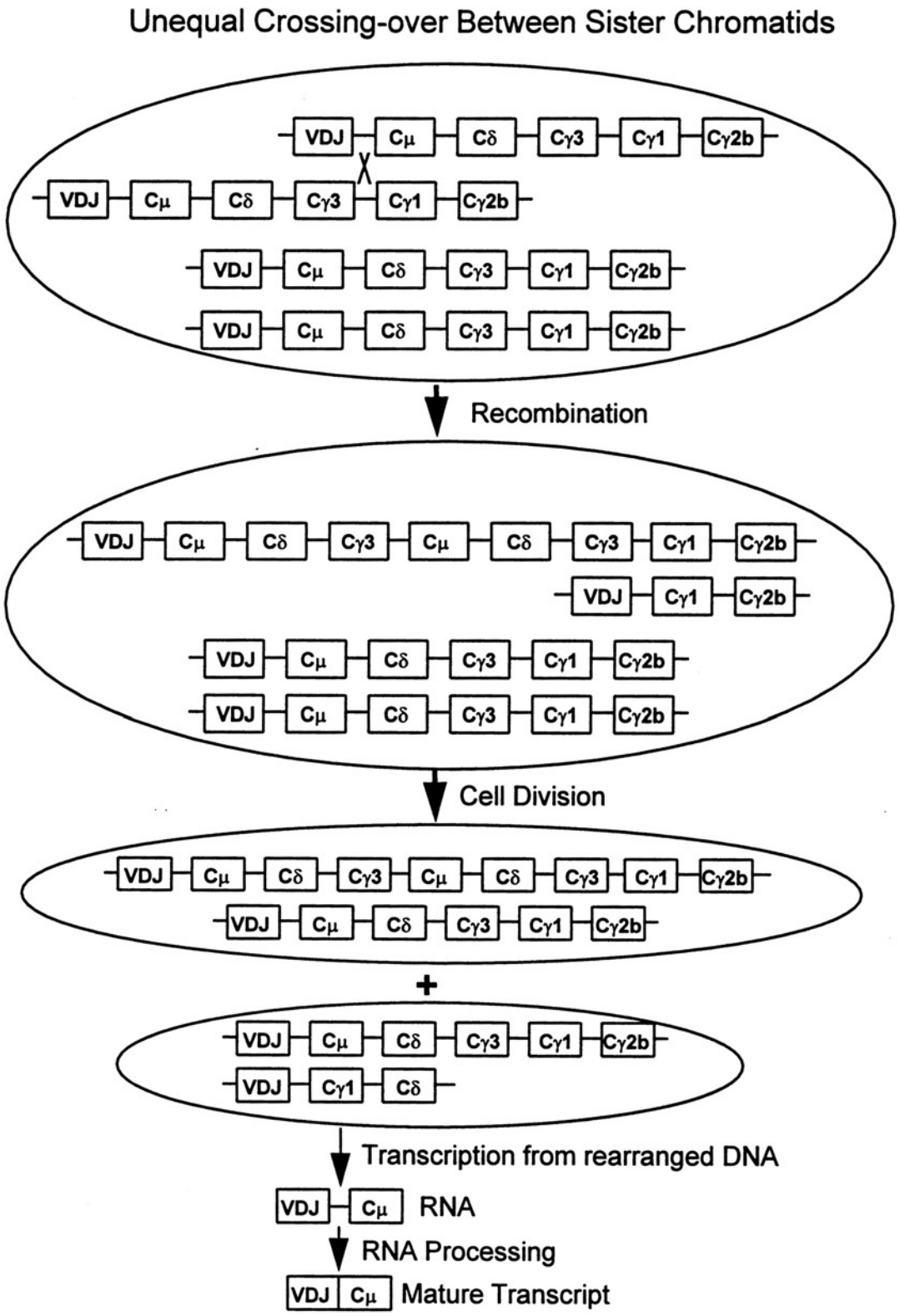


Figure 23. Recombination between two sites on sister chromatids, with one chromatid, and the cell into which it segregated, acquiring the C_H genes lost by it's sister. (Used with permission, reference 41.)

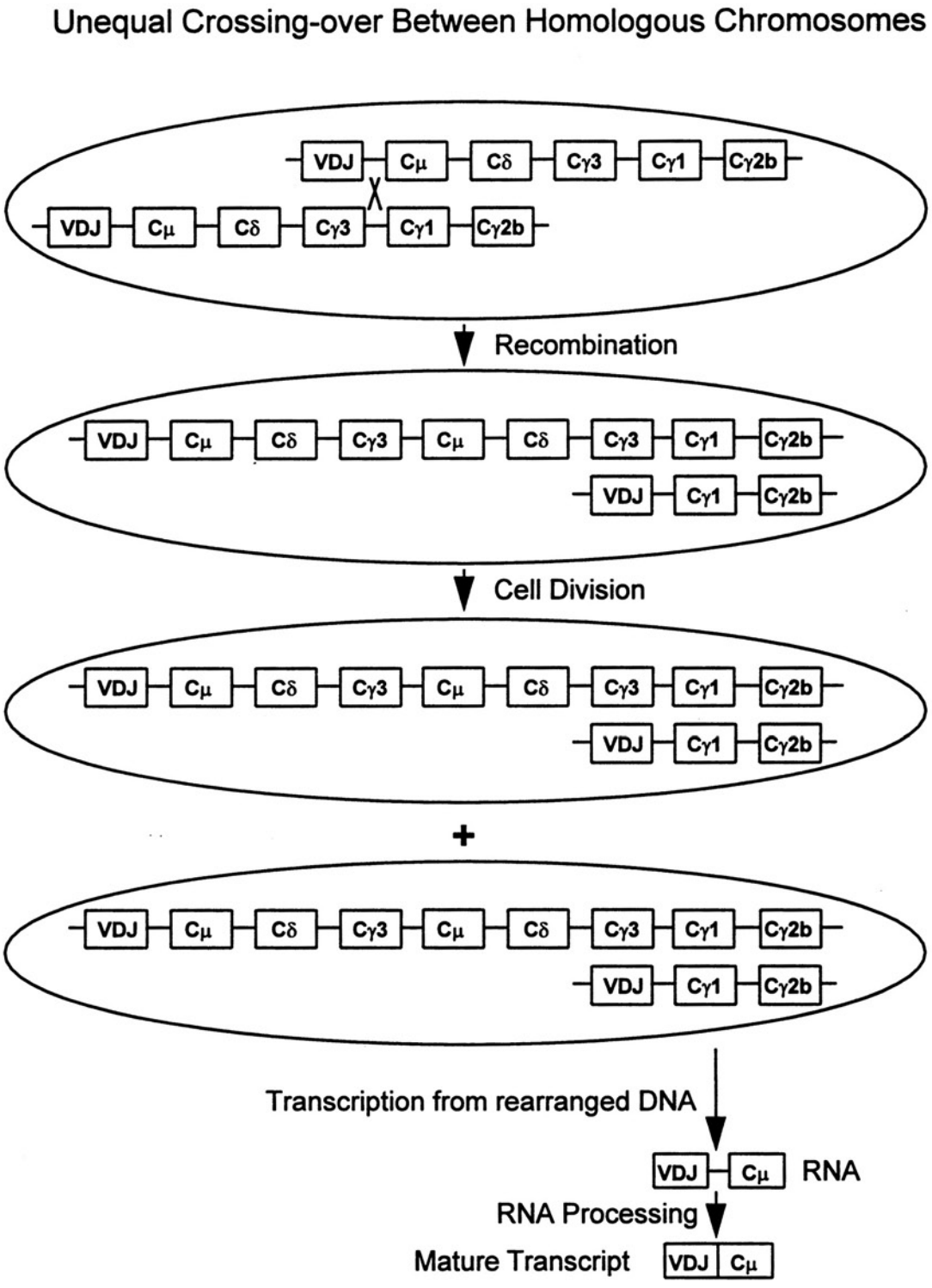


Figure 24. Recombination between two homologous sites on paired chromosomes with one chromosome acquiring the C_H genes that were deleted from its homolog. (Used with permission, reference 41.)

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